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ORIGINAL ARTICLE

The efficacy of luliconazole and caspofungin on planktonic and biofilm of *Candida albicans* from different sources

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ABSTRACT

Background and Objectives: The ability of *Candida albicans* to produce biofilm is considered an important pathogenic factor. In addition, the low sensitivity of biofilms to antifungal drugs is a challenge for patients, clinicians, and laboratory workers. We aimed to investigate the effectiveness of luliconazole and caspofungin on the planktonic and biofilm types of *C. albicans* strains.

Materials and Methods: Fifty *C. albicans* from vaginitis, candiduria, gastrointestinal candidiasis, and saliva were examined for antifungal susceptibility against caspofungin and luliconazole using the CLSI M27 guideline. Moreover, the susceptibility of biofilms was detected using 96 well microplates and the MTT method.

Results: The capacity of the isolates to produce biofilm within 2, 6, and 24 h was different, however, all tested isolates produced biofilm after 24 h. Vaginal and esophagitis isolates had a high and low ability for biofilm production during 24-hour incubation. In our study, 90% of isolates were sensitive to caspofungin, while 7.5 and 2.5% of them were intermediate and resistant. The MIC range of all isolates against luliconazole was 0.01562-1 µg/mL.

Conclusion: The MICs of biofilms were 15.6, and 171.3 higher than that of planktonic cells for caspofungin and luliconazole, respectively. Moreover, paradoxical and trailing effects occurred at 4 and 32 µg/mL of caspofungin and luliconazole, respectively.

Keywords: *Candida albicans*; Biofilm; Planktonic; Luliconazole; Caspofungin

INTRODUCTION

Candida albicans, an important pathogenic yeast, is naturally found in human mucosa, including the oral cavity, pulmonary airways, gastrointestinal, and genitourinary tracts. Although some host factors have an important role in the disease process and its development, microbial factors also have crucial effects. Yeast to hyphal form switching, facilitates the organisms adhesion and penetrate into tissues whereas biofilms leads to chronic disease, spreading to other sites, and resistance development

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to antifungals (1). The planktonic form of *Candida* is usually found as normal mycoflora whereas microbial biofilms are a complex structure of microorganisms embedded in a network of proteins attached to biomaterial surfaces. Many *Candida* species can produce biofilms that include various yeast and budding cells, true hyphae, and pseudohyphae (2). Generally, the ability of *Candida* species to produce biofilm is considered as the main virulence factor (3).

The biofilm antifungal susceptibility is one of the challenges in treating candidiasis. Moreover, several studies revealed that *Candida* in yeast form is highly sensitive to antifungal drugs than the biofilm form (3, 4). For example, *Candida* biofilms are less sensitive to amphotericin B, fluconazole, itraconazole, ketoconazole, and caspofungin than their planktonic forms (4). Zarei-Mahmoudabadi et al., Ferreira et al., Silva et al., and Tobudic et al., studies have shown that the minimum biofilm inhibitory concentration (MBIC) of biofilms of *C. albicans* was highly increased against amphotericin B and fluconazole than planktonic cells (2-5). Moreover, Prażyńska et al., showed that the effectiveness of antifungals on biofilm varies at different stages of its production and is time-dependent (6). For example, the early stages of biofilm maturation (2 h) are more sensitive to antifungal than 6 h-old biofilms, and 24 h-old biofilms are more resistance than other biofilm stages. Several bio-medical devices, such as a central venous catheter, shunts, artificial knees, breast implants, tissue heart valves, and dentures get infected by *Candida* biofilms (5). Moreover, the biofilm complex of *Candida* acts as a reservoir source of the organism for further distribution in the host (5).

Caspofungin is a member of the echinocandins antifungal class with fungicidal activity against most *Candida* species. Caspofungin is prescribed as an effective first-line therapy for invasive candidiasis by researchers (7, 8). Luliconazole, an imidazole antifungal, has high potency against several fungal species, especially *Candida* species (9-13). However, only the topical preparations of luliconazole are available in some countries. In the present study we investigated the biofilm development and effectiveness of luliconazole and caspofungin on the planktonic and different biofilm production stages by *C. albicans* isolates from clinical and environmental sources.

MATERIALS AND METHODS

Isolates sources. In this study, 50 *C. albicans* strains from clinical and environmental sources were examined, including 10 isolates from vaginitis (14), 10 isolates of candiduria (15), 10 isolates of gastrointestinal candidiasis (16), and 10 isolates of healthy people saliva (17). The used *C. albicans* had been previously detected using PCR-RFLP during previous projects. A suspension of each yeast in distilled water was kept at ambient temperature at the department of Medical Mycology affiliated with Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Moreover, 10 environmental isolates of *C. albicans* were also isolated from the different areas in Ahvaz. Moisture swabs were used for sampling from environmental surfaces, inoculated on CHROMagar Candida (CHROMagar *Candida*, France) plates, and incubated at 35ºC. Isolates were screened based on green colonies production, then confirmed using morphological methods (germ tube production on serum at 37ºC, and chlamydoconidia production on cornmeal agar at 29ºC.

Antifungal susceptibility assay of *Candida albicans* planktonic form. The susceptibility of planktonic form isolates against antifungals was assessed based on the CLSI M27 guideline (18). All tested isolates were sub-cultured on Sabouraud dextrose agar, SDA (Biolife, Italia), and incubated at 35°C for 24 hrs. A standard suspension of yeasts (0.5 McFarland) was prepared in sterile distilled water. Then, suspensions were diluted at 1:20 and 1:50 in RPMI 1640 (Gibco, UK). Luliconazole (API Chem Technology, China) and caspofungin (Sigma - Aldrich, Germany) were prepared at serial concentrations, from 2 to 0.0078 µg/mL in RPMI 1640. Finally, 100 µL of serial dilutions of each antifungal and 100 µL of the standard suspension were mixed in 96 microplate wells and incubated at 35°C for 24 hrs. The minimum inhibitory concentration (MIC), MIC_{50}, MIC_{90}, and MIC geometric (GM) of antifungals for each group of isolates were calculated.

Antifungal susceptibility assay of *Candida albicans* biofilm form. The antifungal susceptibility of biofilms were performed according to Prażyńska1 et al. (6). The isolates were inoculated on SDA and incubated at 37°C overnight. Then, a colony of each isolate was inoculated at 20 mL yeast nitrogen base, YNB (Fluka, Germany) supplemented with 0.25%

glucose (PanReac, Spain). Culture media were incubated at 37°C at 120rpm for 24 h. Cultures were centrifuged at 3000 g for 5 min, and washed three times with sterile PBS. Yeast cells were re-suspended in at least 5 mL of YNB and standardized to 0.5 McFarland. Each well of 96-well flat-bottomed microplates (Biofil, China) was filled with 100 µL of standard suspensions. The negative control well contained 100 µL sterile YNB. All procedures were performed in triplicate. The microplates were incubated at 37°C for 2, 6, and 24 h for biofilm production at different stages of maturation. The suspensions of the first, second, and third sets of microplates were discarded after 2, 6, and 24 h of incubation, respectively, and then wells were washed three times in sterile PBS and filled with 100 µL of serial dilutions of luliconazole (1-32 µg/mL) and caspofungin (0.125-4 µg/mL) in RPMI 1640. Positive and negative controls included an untreated biofilm and an empty well, respectively. Both controls were inoculated with 100 µL of free drug-RPMI 1640. Finally, after 24 h of incubation at 35°C, wells contents were removed and completely rinsed three times with sterile PBS.

MTT reduction assay. In the present study, MTT (3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) test was used for the quantitation of antifungal susceptibility *Candida* biofilms (6). The MTT cell viability assay kit (DNAbiotech, Tehran) was used as described by the manufacturer's manual. Then microplate was read using the ELISA reader at 570 nm and ODs were recorded. The ability of biofilm formation was calculated according to Tulasidas et al, (19). The biofilm formation was categorized into 4 types as follows; OD ≤ ODC = non-adherent, ODC < OD ≤2 ODC = weakly adherent, 2ODC < OD ≤ 4 ODC = moderately adherent, and 4ODC < OD = strongly adherent.

Statistical analysis. In the present study, the MIC range of isolates for antifungals was tabulated then MIC_{50}, MIC_{90}, and MIC_{GM} for each *Candida* set as well as all tested isolates were calculated. The adherent activity for each *Candida* set was calculated based on Tulasidas et al. (19) and categorized as non-adherent, weakly, adherent, and strongly adherent. The results were presented as a chart using Excel (Office 16 software). Moreover, the results of biofilm antifungal susceptibility against caspofungin and luliconazole were presented as a chart.

RESULTS

Biofilm formation of *Candida albicans.* The biofilm formation of 50 isolates of *C. albicans* recovered from clinical and environmental sources was examined. As shown in Fig. 1, biofilm formation was observed after 2, 6, and 24 h of incubation in all isolates, whereas only two isolates (4%) (one isolate from each oral and, urine samples) were negative for biofilm after 2 h. In addition, one isolate from the esophagitis sample was unable to form biofilm after 24 h. Most of the vaginal isolates had a strong ability to produce biofilm after 2, 6, and 24 h of incubation. In contrast, the lowest ability for biofilm production was attributed to environmental isolates of *C. albicans*. The details of biofilm production in each group of isolates are provided in Fig. 1. The growth curve using the MTT assay absorbance was related to the biofilm density of the isolates.

Susceptibility of planktonic of *C. albicans* against caspofungin and luliconazole. The susceptibility of 50 *C. albicans* in planktonic and biofilm forms was evaluated against two antifungals, caspofungin and luliconazole. Table 1 shows the minimum inhibitory concentration (MIC) range of all tested isolates for caspofungin ranked from 0.0625 to 1 µg/mL with MIC_{50}, MIC_{90}, and MIC_{GM} 0.125, 0.25, and 0.12851 µg/mL.

Susceptibility of biofilm of *C. albicans* against caspofungin and luliconazole. Although, the planktonic form of *C. albicans* is more sensitive to antifungal agents, a high resistance rate to antifungals (up to 1000-fold higher than planktonic types) was reported

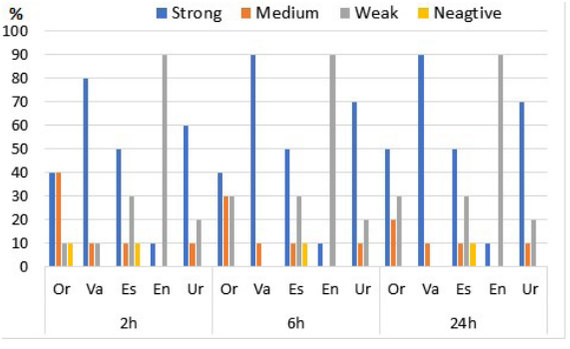

Fig. 1. Biofilm production among vaginal (Va), urine (Ur), esophagitis (ES), oral (Or), and environmental (En) isolates of *Candida albicans* after 2, 6, and 24 hours

Table 1. The antifungal susceptibility of 50 isolates of *Candida albicans* (yeast form) to luliconazole and caspofungin.

Isolates sources (No.)	Antifungals	S (≤0.25)	I (0.5)	R (≥1)	Minimum inhibitory concentration (MIC)			
					MIC range	MIC_{50}	MIC_{90}	MIC_{GM}
Vaginal (10)	Luliconazole	-	-	-	0.0312-0.25	0.625	0.25	0.08244
	Caspofungin	9 (90%)	-	1 (10%)	0.0625-1	0.125	0.25	0.18946
Candiduria (10)	Luliconazole	-	-	-	0.0312-0.25	0.0625	0.125	0.07691
	Caspofungin	10 (100%)	-	-	0.0625-0.25	0.0625	0.25	0.08247
Esophagitis (10)	Luliconazole	-	-	-	0.01562-0.625	0.0312	0.0625	0.03346
	Caspofungin	10 (100%)	-	-	0.0625-0.25	0.125	0.25	0.125
Oral cavity (10)	Luliconazole	-	-	-	0.01562-1	0.25	1	0.26793
	Caspofungin	7 (70%)	3 (30%)	-	0.0625-0.5	0.125	0.5	0.14359
Environmental (10)	Luliconazole	-	-	-	0.125	0.125	0.125	0.125
	Caspofungin	10 (100%)	-	-	0.0625-0.25	0.125	0.25	0.125
Total (50)	Luliconazole	-	-	-	0.01562-1	0.125	0.25	0.09339
	Caspofungin	46 (92%)	3 (6%)	1 (2%)	0.0625-1	0.125	0.25	0.12851

S: Sensitive; I: Intermediate; R: Resistant
Breakpoints for caspofungin: Sensitive ≤ 0.25 µg/mL; Intermediate = 0.5 µg/mL; Resistant ≥1 µg/mL
No defined breakpoints for luliconazole.

for their biofilms (2, 3, 5). The details of the sensitivity of the biofilm of *C. albicans* strains to different concentrations of caspofungin (0.125-4 µg/mL) are shown in Fig. 2. As shown in Fig. 2, after 2, 6, and 24 hrs of biofilm formation and being exposed to the caspofungin, with increasing antifungal concentration from 0.125 to 2 µg/mL, the trend of biofilm production was decreased. In the present study the MIC of biofilms (MIC biofilms, 2 µg/mL) was 15.6 higher than that of planktonic cells (MIC_{GM} planktonic cells, 0.12851 µg/mL). The details of the sensitivity of the biofilm of *C. albicans* isolates to various concentrations of luliconazole (1-32 µg/mL) are shown in Fig. 2. As shown in Fig. 2, after 2, 6, and 24 hrs of biofilm formation and being exposed to the luliconazole, with

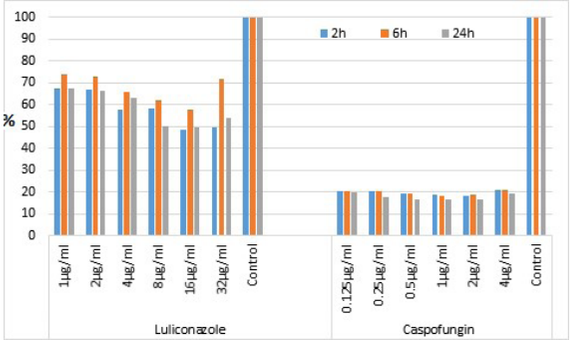

Fig. 2. The effect of serial concentrations of caspofungin and luliconazole on *Candida albicans* biofilms

increasing antifungal concentration from 1-16 µg/mL, the trend of biofilm production was decreased.

DISCUSSION

Candida albicans can switch to different forms (Polymorphic) in suitable conditions and each form has variable virulence capacity. The planktonic form of *C. albicans* (yeast cell form) usually colonizes mucocutaneous tissues as normal mycoflora, whereas mycelial forms are considered pathogen form that invades different tissues (1). Another form, biofilm, is a complex of yeast cells, hyphal form, and embedded matrix that is performed on biomaterial devices (20, 21). Biofilm production along with extracellular enzymes are the main pathogenicity factors for the invasion of *Candida* species into tissues (5). High-biofilm-producing *Candida* species are associated with body fluids, biopsy, and catheter isolates, whereas *Candida* strains from candiduria and pulmonary system have low-biofilm-producing potential (22). Moreover, Zarei Mahmoudabadi et al., study showed that the vaginal isolates of *C. albicans* have the highest degree of biofilm production followed by candiduria and oral isolates (2). Similarly, in our study, vaginal isolates were more capable of biofilm production at different times (2, 6, and 24 h), followed by urinary and oral isolates. The biofilm growth

curve demonstrates the increased colorimetric reading obtained by the MTT-reduction assay (OD) correlated with biofilm production. As shown, the highest and lowest potential for biofilm production is associated with vaginal and environmental isolates, respectively.

According to the CLSI M60 guideline (23), 46 (92%) of isolates were sensitive to caspofungin (MIC ≤0.25) whereas only 3 (6%) and 1 (2%) were intermediate and resistant, respectively. Caspofungin, an inhibitor of 1,3 βD glucan synthase, has been described as an effective invasive candidiasis antifungal (8). Moreover, several reports have shown that the MIC of caspofungin against *C. albicans* ranged from 0.015 to 1.0 µg/mL (24-26). In a study by Shokohi et al. 95.4% of *C. albicans* strains were sensitive to caspofungin (25) whereas, no resistant isolate was found by Yenisehirli et al., (26). Luliconazole is a new azole antifunga that has been studied against several species of yeasts and molds (9, 11, 12, 27-29). The very low MIC of luliconazole against *Candida* species is one of its remarkable properties. In the present study, the MIC range, MIC_{50}, MIC_{90} and MIC_{GM} luliconazole for 50 isolates were 0.01562 - 1 µg/mL, 0.125, 0.25, and 0.09339 µg/mL, respectively.

Caspofungin (2 µg/mL) was presented as a good candidate for the fungal biofilms control (3). Also, in this study, it was found that the paradoxical effect (Eagle effect) was observed at 4 µg/mL of caspofungin. A study showed that in the high concentration of echinocandins, biofilm of *Candida* displays paradoxical grow, however the clinical significance of the phenomena is unclear (3). Several studies show that luliconazole is highly effective in *C. albicans* planktonic form in vitro, however, we couldn't find the luliconazole efficacy on the *Candida* biofilm for comparison. In this study, the MIC of *Candida* biofilms was 171.3 higher than that of planktonic cells (MIC_{GM} planktonic cells, 0.09339 µg/mL/biofilm MIC, 16 µg/mL). Also, in this study, it was found that the trailing effect (persistent growth) was observed at 32 µg/mL of luliconazole. The trailing effect is an in vitro phenomenon observed when *Candida* species were tested against high concentrations of azoles (30). It seems that trailing effect in *C. albicans* reflects azole tolerance.

Limitations. The limitation of this study was that other antifungals were not used against biofilm. Moreover, we only tested 50 *C. albicans* strains from 5 different sources. For better results, more isolates from other clinical specimens and other antifungals are recommended.

CONCLUSION

In summary, the biofilm formation by *C. albicans* isolates could depend on the sample sources. So, most of the vaginal isolates had a strong ability for biofilm production, and the lowest ability for biofilm production was attributed to environmental isolates. Although both antifungals were effective on the isolates at low concentrations, the MIC of biofilms was 15.6 and 171.3 higher than that of planktonic cells for caspofungin and luliconazole, respectively. Moreover, it was found that the paradoxical and trailing effects were observed at 4 µg/mL of caspofungin and 32 µg/mL of luliconazole.

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