Microbes and Infectious Diseases 2022; 3(1): 176-184

Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Candida albicans blastospores and hyphae respond differentially to fluconazole: additional virulence factor of germination process

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ARTICLE INFO

Article history:
Received 28 September 2021
Received in revised form 22 October 2021
Accepted 3 November 2021

Keywords:
Candida albicans
Fluconazole
Germination
Hyphae
Virulence

ABSTRACT

Background: The morphologic transition from yeasts (blastospores) to hyphae, through the germination process, is a crucial virulence factor of *Candida albicans (C. albicans)*, as it enhances adherence, tissue invasion and biofilm formation. The study aimed at comparing fluconazole susceptibility patterns of *C. albicans* yeast and germinated forms. **Methods:** Six *C. albicans* strains, including *C. albicans* ATCC 10231 and 5 clinical isolates were tested in yeast forms and germinated forms for fluconazole susceptibility using CLSI M27-A3 broth microdilution and CLSI M44-A2 disk diffusion reference methods. **Results**: Fluconazole minimal inhibitory concentrations (MICs) were 0.125-1 µg/ml and 0.25-4 µg/ml for yeasts and germinated forms, respectively. The sensitivities of yeasts were higher than those of their corresponding germinated forms with 1 to 4-fold dilutions differences. The MIC_{50}s of yeasts and germinated forms were 0.125 and 1 µg/ml, respectively, with 3-fold dilution difference, while MIC_{90}s were 1 and 4 µg/ml for yeasts and germinated forms, respectively, with 2-fold dilution difference. Means of inhibition zones were 31-46.7 mm and 29.3-43.7 mm for yeasts and germinated forms, respectively. The germinated forms of all tested strains had inhibition zones smaller than those of their corresponding yeast forms with 0.2-4.8 mm differences. There was significant statistical difference in sizes of inhibition zones between yeasts and germinated forms (p = 0.043). **Conclusions:** Yeast and hyphae of *C. albicans*, both in planktonic state, respond differentially to fluconazole, as the hyphae are less sensitive than their yeasts counterparts, which can be considered as a virulence factor for hyphae, which should be considered in treatment plans of candidal infections.

Introduction

Candida albicans (C. albicans) is one of the most common opportunistic fungal pathogens affecting humans all over the globe and causes cutaneous, mucosal as well as invasive infections, involving blood stream infection (candidemia) and disseminated infections [1,2].

In last decades, candidemia and other candidal infections have become more prevalent due marked increase in immunocompromisation due to diabetes mellitus, misuse of broad-spectrum antibiotics, malignancies and anticancer chemotherapy, increased use of immunosuppressive drugs and interventional procedures in hospitals

DOI: 10.21608/MID.2021.98513.1198
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especially intravenous catherization [3,4]. Indeed, candidal deep seated infections act as challenges for health care systems due to their high mortality rates [2,5].

Candida albicans grows in different morphologic forms including unicellular yeasts (blastospores), pseudohyphae and true hyphae, and can characteristically switch reversibly between these forms. The transition in morphogenesis from yeast to hyphal forms occurs through the germination process which is controlled by different genes [6,7], that are induced by transcription factors resulted from activation of different signal transduction pathways [8] in response to different environmental stimuli including nutrients, temperature, pH changes, oxygen and carbon dioxide tensions [9-12]. Additionally, internal signals originating from inside the yeast cells were found to regulate the hyphal development [13].

The germination process is an important pathogenicity and virulence factor for *C. albicans*, as the resultant hyphal forms enhance adherence to target surfaces, tissue invasion and biofilm formation [14,15]. Many genes and proteins involved in hyphal development were proved to be co-involved in virulence as well [16,17]. Moreover, the germination has immunomodulatory effects that evade host immune defense, as the germinated forms of *C. albicans* were found to suppress and inhibit IL-12 production by monocytes [18], in addition to inhibitory and killing effects on phagocytes [19,20].

Overall, the modulation in morphogenesis enables this pathogen to survive and set its pathogenicity under different growth and environmental conditions [21]. The ability of *C. albicans* to reversibly switch between different morphologic forms is a crucial virulence determinant, as the different candidal morphologic forms behave differently in pathogenicity processes and immune interactions, due to structural and growth behavioral differences between these forms [22-24].

Depending on this, it is anticipated that the response to antifungal drugs may differ between different candidal growth forms. Indeed, triazole antifungals, including fluconazole, are among the most widely used drugs for treatment of candidal infections worldwide, and the resistance to such drugs is a true challenge. Generally, candidal azole resistance may be either intrinsic due to inherent fungal structural factors or acquired through development of different resistance mechanisms [25-27]. So, the morphogenesis differences between candidal growth forms may confer them structural and/or functional characters that may alter their response behavior to antifungal agents.

This study aimed at comparing the antifungal response of two different morphologic forms of *C. albicans*, by determining the susceptibility patterns of yeast and germinated forms for fluconazole, to investigate if the germination process and morphologic transition from yeast to hyphae could add to the virulence of this fungal pathogen regarding response to antifungals, which consequently, could help in modification of current treatment strategies of *C. albicans*.

Materials and Methods

Test strains

Six *C. albicans* strains were tested, including *C. albicans* ATCC 10231 as a reference strain and 5 clinical isolates, which were isolated by culturing of the clinical specimens swabs obtained from 2 cases of vaginitis and 3 cases of oral candidiasis on Sabouraud dextrose agar (SDA) (Oxoid), then identified using Gram staining, germ tube formation in 10% (vol/vol) fetal bovine serum (GIBCO, Invitrogen, Auckland, NZ) in phosphate buffered saline (PBS), and chlamydospore formation by subculturing on corn meal agar (Himedia, India), and finally, the identification was verified by VITEK 2 automated system using VITEK 2 YST ID Card (BioMérieux, France) according to manufacturer instructions.

Antifungal susceptibility testing

All tested strains were tested in yeast forms and germinated forms, both in planktonic states, against fluconazole using broth microdilution and disk diffusion methods.

Inoculum preparation (Test forms)

Yeast forms: The tested strains were subcultured on SDA and incubated at 35ºC for 24 hours. Then, 3-5 colonies were collected using sterile disposable loop and resuspended in 5 ml of sterile isotonic saline, vortexed for 15 seconds, and then the turbidity was adjusted using spectrophotometer at wavelength 530 to 0.5 McFarland standard to obtain cell suspension of $1\text{-}5\times10^6$ CFU/ml. This inoculum was used for disk diffusion testing, while in broth microdilution method, it was diluted 1:1000 to obtain $1\text{-}5\times10^3$ CFU/ml, which was 2× final desired inoculum.

Germinated forms: The germination was induced using fetal bovine serum (GIBCO, Invitrogen, Auckland, NZ). First, test strains were subcultured on SDA for 24 hours at 35ºC, then, 1-3 colonies were collected using sterile disposable loop and resuspended in 3 ml volumes of 10% (vol/vol) fetal bovine serum in phosphate buffered saline (PBS), incubated at 35ºC for 200 minutes on a rocker,

examined regularly every 25 minutes to check germ tube formation. The germ formation was considered when the length of the germ tube is equal or more than the width of the yeast cell. Then, the germinated forms were harvested by centrifugation, decanting the supernatant and washing the pellets in PBS three times. Then, the pellets were resuspended in 5 ml of sterile isotonic saline, vortexed for 15 seconds and the turbidity was adjusted to 0.5 McFarland standard using spectrophotometer at 530 nm wavelength to obtain cell suspension of $1\text{-}5\times10^6$ CFU/ml. This inoculum was used for disk diffusion testing, while in broth microdilution method, it was diluted 1:1000 to obtain $1\text{-}5\times10^3$ CFU/ml, which was 2× final desired inoculum.

Antifungal agent

The antifungal susceptibilities of test strains were tested for fluconazole. In broth microdilution method, fluconazole (Sigma-Aldrich) was prepared according to clinical and laboratory standards institute (CLSI) M27-A3 method guidelines [28], to obtain 96-well plates of RPMI 1640 medium (GIBCO, Invitrogen, Auckland, NZ) containing 2× desired final concentrations 0.125 µg/ml to 64 µg/ml for fluconazole. In disk diffusion method, fluconazole 25 µg disks (Oxoid) were used.

Broth microdilution method

It was performed according CLSI M27-A3 method guidelines [28]. For each test strain, 100 µl volumes of the prepared 2× inoculum suspensions of yeast form and germinated form were added to the wells of the previously prepared fluconazole 96-well plates to obtain final desired concentration of $0.5\text{-}2.5\times10^3$ CFU/ml . Separate plates were used for each candidal form. Sterility and growth controls were run for each tested isolate. Each strain was tested in triplicates. The plates were incubated at 35ºC for 48 hours. For each tested isolate, minimal inhibitory concentration (MIC) (the lowest concentration of fluconazole inhibited ≥50% of growth compared to growth control) was determined visually. For all tested isolates, MIC ranges, MIC_{50} (the lowest concentration of fluconazole inhibited 50% of tested isolates) and MIC_{90} (the lowest concentration of fluconazole inhibited 90% of tested isolates) were determined.

Disk diffusion method

It was carried out according to CLSI M44-A2 [29]. For each tested strain, Muller-Hinton agar plates were inoculated by the previously prepared inocula $1\text{-}5\times10^6$ CFU/ml of yeast and germinated forms using sterile swabs, incubated at 35ºC for 24-48 hours. Fluconazole inhibition zones were measured in millimeters. Separate plate sets were used for each candidal form. Each strain was tested in duplicates and the test was repeated on two different occasions.

Data analysis

For broth microdilution method, MIC, MIC_{50} and MIC_{90} were determined for fluconazole against yeast forms and germinated forms of all tested strains. Comparison of fluconazole susceptibilities between yeast and germinated forms was carried out by calculating fluconazole time-fold dilution difference between the MIC, MIC_{50} and MIC_{90} values for both growth forms. For disk diffusion method, the measured fluconazole inhibition zones (in millimeters) were expressed in mean±standard deviation (Mean±SD) for each tested strain, and the results were compared between the two tested growth forms using independent two samples t test. *p* value <0.05 was considered statistically significant. The data was handled and processed using Microsoft Excel 2019.

Results

In this study, CLSI M27-A3 broth microdilution and CLSI M44-A2 disk diffusion methods were used to compare fluconazole susceptibilities between yeast and germinated forms of *C. albicans*.

All strains were tested both in yeast forms (blastospores) and in hyphal forms after induction of germination by fetal bovine serum for sufficient time periods as shown in **figure (1)**.

Broth microdilution

All tested strains were sensitive to fluconazole according to interpretive breakpoints of CLSI M27-A3 for *Candida* species and fluconazole, as the highest MICs obtained by tested yeast and germinated forms were 1 µg/ml and 4 µg/ml, respectively. For the yeast forms, fluconazole MIC ranged from 0.125 to 1 µg/ml, as three clinical isolates had MICs of 0.125 µg/ml, the other two clinical strains gave MIC of 0.25 µg/ml, while the reference strain *C. albicans* ATCC 10231 gave higher MIC (1 µg/ml). For the germinated forms, fluconazole MIC range was 0.25-4 µg/ml, with the lowest MIC (0.25 µg/ml) obtained by clinical strain 5 and the highest MIC (4 µg/ml) by the *C. albicans* 10231 reference strain. It was noted that the variation in fluconazole susceptibility patterns within tested germinated forms was more than within tested yeast forms. Overall, the sensitivities of the yeast forms were higher than those of their corresponding germinated forms with 1 to 4-fold dilutions differences in fluconazole MICs. The MIC_{50}s of yeast forms and germinated forms were 0.125 and 1 µg/ml, respectively with 3-fold dilution difference, while MIC_{90}s were 1 and 4 µg/ml for

yeast forms and germinated forms, respectively, with 2-fold dilution difference **(Table 1)**.

Disk diffusion

All tested strains were sensitive to fluconazole according to zone diameter interpretive criteria of CLSI M44-A2 for *Candida* species and fluconazole. As shown in **table (2)**, the tested clinical strains had inhibition zones' means ranging from 40.7 mm to 46.7 mm in diameter, while *C. albicans* ATCC 10231 inhibition zone mean was 31 mm in diameter. The germinated forms of all tested strains had inhibition zones smaller than those of their corresponding yeast forms with differences of 0.2 mm, 4.8 mm, 4.7 mm, 3 mm, 3 mm and 1.7 mm for clinical strains 1, 2, 3, 4, 5, and *C. albicans* ATCC 10231, respectively. Overall, there was significant statistical difference in sizes of inhibition zones between yeast forms and germinated forms (p = 0.043).

Figure 1. Wet mount preparations for the growth forms of tested *Candida albicans* strains. A&B, yeast forms (blastospores) showing round to oval cells with budding. C-J, germinated and hyphal forms after incubation in 10% (vol/vol) fetal bovine serum in phosphate buffered saline for different time periods, maximum for 200 minutes; C-F show germ tubes with different sizes, G-J show hyphae with variable sizes (Magnification 400×).

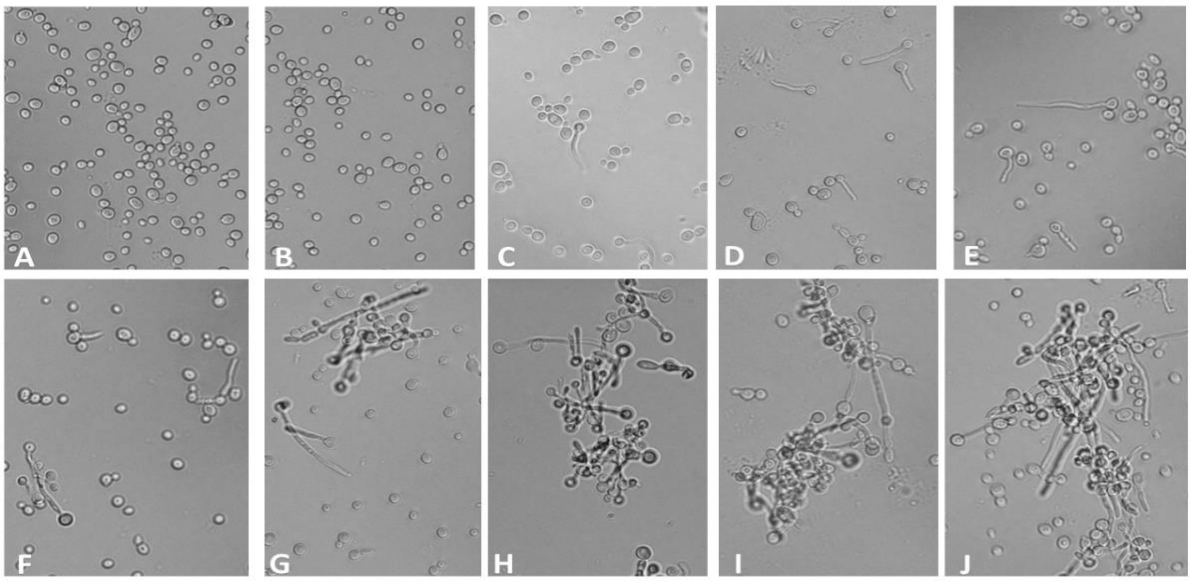

Table 1. Fluconazole minimal inhibitory concentration (MIC) values for yeast forms and germinated forms of tested *Candida albicans* strains obtained from broth microdilution method.

***C. albicans* strain**	**Fluconazole MIC*(µg/ml)**		**Time-fold dilution difference**
	Yeast form	**Germinated form**	
Clinical strain 1	0.25	1	2
Clinical strain 2	0.25	2	3
Clinical strain 3	0.125	2	4
Clinical strain 4	0.125	1	3
Clinical strain 5	0.125	0.25	1
ATCC 10231	1	4	2
MIC range	0.125-1	0.25-4	1-4
MIC_{50}**	0.125	1	3
MIC_{90}***	1	4	2

*MIC the lowest concentration of fluconazole inhibited ≥50% of growth of a tested isolate compared to its growth control.
**MIC_{50} the lowest concentration of fluconazole inhibited 50% of tested isolates.
***MIC_{90} the lowest concentration of fluconazole inhibited 90% of tested isolates.

Table 2. Fluconazole inhibition zones (measured in mm) for yeast forms and germinated forms of tested *Candida albicans* strains obtained from disk diffusion method.

***C. albicans* strain**	**Fluconazole inhibition zones (mm) Mean±SD**		***p* value***
	Yeast form	**Germinated form**	
Clinical strain 1	40.7±2.16	40.5±0.55	0.043
Clinical strain 2	44±1.58	39.2±1.09	
Clinical strain 3	44.7±1.53	40±1	
Clinical strain 4	42.5±2.08	39.5±1.29	
Clinical strain 5	46.7±3.05	43.7±1.53	
ATCC 10231	31±1	29.3±0.71	

***** *p* value calculated by independent two samples t test between inhibition zones' dimeters of yeast forms group and germ forms group.

Discussion

The current study investigated impact of *C. albicans* morphologic transformation from yeast to hyphae on response to fluconazole by comparing its susceptibility patterns for both yeast and germinated (hyphal) growth forms.

The hyphae play a basic role in virulence of *C. albicans* and constitute a major element in structure of candidal biofilms, which are characterized by high resistance to many antifungals, that can reach up to 1000-fold when compared to planktonic cells [30]. In this context, many studies investigated the role of hyphal forms in biofilm state, in conjunction with other constituents of biofilms like extracellular matrix, the current study differs from those studies in testing the hyphal forms in planktonic state, not in biofilm state, to investigate the effect of morphological switch from yeast to hyphae on the virulence regarding the behavior of hyphal form itself against antifungals.

In the study, both broth microdilution and disk diffusion testing revealed that all tested strains of *C. albicans* strains were sensitive to fluconazole, but the tested yeast and hyphal forms of the same *C. albicans* strains had different antifungal responses as the germinated forms were less sensitive to fluconazole than the yeast forms.

This differential response between yeast and hyphal forms comes in concordance with the results of a previous study [31] that revealed differential expression of many proteins during transition process of *C. albicans* from yeast to hyphae in response to the antifungal HWY-289. These proteins were involved in regulation of cell cycle, metabolic processes, cellular defenses and signaling pathways.

The higher fluconazole sensitivity of yeasts than hyphae, shown by the current study, may originate from the differential action and adaptive responses of the antifungal on the candidal growth form. This differential antifungal response agrees with the findings of the study carried out to explain the mechanism of action of the antifungal HWY-289. This antifungal was found to inhibit candidal transition from yeast to hyphae due to its differential action on these candidal growth forms, as HWY-289 downregulated two functional protective proteins and induced expression of three RAS-related genes in yeast forms only, not in hyphal forms [31], rendering this antifungal more active against the yeast forms than the hyphal forms. Another study about proteomic analysis revealed changes in protein abundance in *C. albicans* in response to amphotericin B, one of these proteins, called thioredoxin peroxidase (Tsa1p), was more abundant in hyphal forms than in yeast forms. This protein is an antioxidant enzyme that has a role in oxidative stress by its ability to reduce oxygen reactive forms [32] which may explain, in part, the more durability of hyphal forms than yeast forms in response to antifungals.

There is a difference in physical characters between hyphae and yeast forms of *C. albicans*, as the hyphae have their unique physical properties including morphogenesis, pattern of growth and elongation, directionality and adhesion, so that the hyphae play a major role in virulence of this fungus [33]. These physical properties may influence the interaction of the hyphae with the antifungals conferring the hyphal forms more resistance against antifungals.

One of the major determinants for the fluconazole susceptibility is the phospholipid and

sterol lipid content of the *C. albicans* cell membranes especially ergosterol which act as the target for action of azole drugs, as fluconazole inhibits lanosterol 14α-demethylase enzyme ending by inhibition of ergosterol synthesis. Alterations in composition of cell membrane lipids can lead to decreased antifungal susceptibilities [34,35]. In vitro, the amount of ergosterol in fungal cell membranes varies according to environmental conditions, developmental stage and even the age of the culture [36,37]. Presumably, the larger cell mass and surface area of hyphae compared to their yeast forms counterparts could result in formation of more cell membranes mass necessitating synthesis of larger amounts of ergosterol formed by more lanosterol 14α-demethylase enzyme, so in exposure to the same concentrations of fluconazole, the hyphae may express decreased sensitivity to fluconazole than yeasts due to excess of this enzymes over the binding capacity of the drug, resulting in free enzyme that can complete the ergosterol biosynthetic pathway.

The difference in cell wall between the yeast form and hyphae could be also responsible for the differential response found in the study. Although the cell wall of both candidal forms contain the same types of compounds namely, chitin, glucan, mannans and glycoproteins, the content of chitin layer in three times more in hyphae than in yeast [38,39]. This difference in cell wall may affect the diffusion of fluconazole, and consequently affects its availability at its site of action, resulting in different patterns of response.

Indeed, the hyphal formation occurs through germination or filamentation process and acts as a major virulence factor of *C. albicans* playing major roles in adhesion, invasion, biofilm formation and immune evasion. Additionally, the filamentation process was evidenced to have a protective role against the action of certain antifungal agents, as in the same *C. albicans* strains, the hyphal forms resist the programmed cell death induced by amphotericin B and caspofungin more than the yeast forms, and this protective action was mediated through *MCA1* yeast metacaspase [40]. This type-specific response provides a good example for differential responses of yeast and hyphal forms of *C. albicans* against antifungals.

In conclusion, fluconazole exerts a differential action against yeast forms and hyphae of *C. albicans* in planktonic state, as the hyphae are less sensitive than yeast forms, which may be due to structural or physical characters of the candidal growth form or different adaptive responses of the drug. The decreased sensitivity to fluconazole can be considered as an additional virulence factor for hyphae, which should be considered in treatment plans of candidal infections.

This study is preliminary and needs to be followed by additional studies to examine the behavior of candidal hyphae against antifungals by including more *C. albicans* strains, additional antifungals from different classes and more sophisticated methodologies including molecular, genetic and proteomic analyses to explore the underlying mechanisms for the differential responses of different candidal morphologic forms to antifungals. The expected results could help in detection of new targets for antifungals, more insights into antifungal resistance mechanisms and modification of the current treatment strategies for infections caused by *Candida*, especially the life-threatening disseminated infections.

Funding: None.

Conflicts of interest

The author declares no conflicts of interest.

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