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ESMA KAYA

HATİCE ÖZBİLGE

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Determination of the effect of fluconazole against *Candida albicans* and *Candida glabrata* by using microbroth kinetic assay*

Esma KAYA, Hatice ÖZBİLGE

Aim: To evaluate the antifungal activity of a model antifungal (fluconazole) against *Candida albicans* (ATCC 90028) and *Candida glabrata* (RSKK 04019) strains using a microbroth kinetic assay based on continuous monitoring of changes in the optical density of fungal growth. Recently, antifungal susceptibility testing has become more important because of the increasing incidence of both fungal infections and antifungal drug resistance.

Materials and methods: The optical growth densities of *C. albicans* and *C. glabrata* in the presence of increasing concentrations of fluconazole were measured every 15 min for 24 h at 35 °C, using a multidetection microplate reader at 405 nm. The turbidimetric growth curves of these strains were obtained, and the minimal inhibitory concentration (MIC) values for fluconazole were determined with this kinetic assay. Furthermore, the conventional broth microdilution method was performed according to Clinical and Laboratory Standards Institute reference method M27-A2.

Results: MIC_{90} values for fluconazole were determined as 0.31 µg/mL for *C. albicans* and 16.32 µg/mL for *C. glabrata*.

Conclusion: Using kinetic procedures, the effects of antifungal drugs on target yeasts can be determined at any desired time-point of the incubation period.

Key words: *Candida*, fluconazole, minimal inhibitory concentration, growth curve, optical density, kinetic assay

Candida albicans ve *Candida glabrata*'ya karşı flukonazolün etkisinin belirlenmesinde mikrobroth kinetik yöntemin kullanılması

Amaç: Bu çalışmada, *Candida albicans* (ATCC 90028) ve *Candida glabrata* (RSKK 04019) suşlarının model antifungal olarak seçilen flukonazole karşı antifungal aktivitesi, fungal üremedeki optik dansite değişikliklerini sürekli olarak izlemeye olanak veren kinetik bir sistemle belirlenmiştir. Antifungal duyarlılık testleri son yıllarda gerek fungal enfeksiyonlardaki artış gerekse artan antifungal ilaç direnci nedeniyle giderek daha önemli hale gelmiştir.

Yöntem ve gereç: *C. albicans* ve *C. glabrata*'nın flukonazolün artan konsantrasyonları varlığında, multi-detection microplate okuyucu kullanılarak 405 nm'de 35 °C'de 24 saat süreyle her 15 dakikada bir optik dansiteleri ölçüldü. Bu kinetik sistemle suşların turbidimetrik üreme eğrileri elde edildi ve flukonazolün minimal inhibitör konsantrasyon (MİK) değerleri belirlendi. Ayrıca CLSI referans yöntem M27-A2'ye göre konvansiyonel sıvı mikrodilüsyon yöntemi uygulandı.

Bulgular: Flukonazol $MİK_{90}$ değerleri *C. albicans* için 0,31 µg/mL; *C. glabrata* için 16,32 µg/mL olarak belirlendi.

Sonuç: Kinetik ölçüm yapan yöntemler kullanılarak antifungal ilaçların mayalar üzerindeki etkileri inkübasyon süresince istenilen herhangi bir zamanda gözlenebilir.

Anahtar sözcükler: *Candida*, flukonazol, minimal inhibitör konsantrasyon, üreme eğrisi, optik dansite, kinetik yöntem

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Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Erciyes University, Kayseri - TURKEY

Correspondence: *Esma KAYA, Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Erciyes University, 38039 Kayseri - TURKEY*
E-mail: esmagkaya@yahoo.com.tr

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Introduction

Recently, antifungal susceptibility testing has become more important because of the increasing incidence of both fungal infections and antifungal drug resistance (1,2). For this reason, the Clinical and Laboratory Standards Institute (CLSI) has developed a reference method for broth dilution antifungal susceptibility testing of yeasts (3). This CLSI document recommends the visual determination of 48-h minimum inhibitory concentration (MIC) values, with the azole MIC being defined as the lowest concentration at which a prominent decrease in turbidity is observed (4,5). However, a visual evaluation of the results is partly a subjective assessment, and it has been seen that the trailing effect of azoles complicates the determination of their MIC values (2,5,6). It has been demonstrated that this decrease in growth (turbidity), as determined by a spectrophotometer, corresponds to approximately 50% inhibition in growth (4,5).

The turbidimetric growth curves of yeasts and molds in the presence of antifungal agents can be obtained by using a spectrophotometric kinetic assay based on continuous monitoring of changes in the optical density (OD) of fungal growth (7,8). Using this assay, the MIC values of drugs can be determined in a shorter period than with conventional methods. Results can also be detected during exposure and are more objective because spectrophotometric MICs are determined instead of visual MICs. Thus, using kinetic procedures, the effects of antifungal drugs on target yeasts can be determined at any desired time-point of the incubation period (7-9). In this study, we aimed both to describe the turbidimetric growth curves of *Candida albicans* and *Candida glabrata* in the presence of increasing concentrations of a model antifungal (fluconazole) and to determine the MIC by using this system.

Materials and methods

Strains and preparation of inocula: *C. albicans* (American Type Culture Collection (ATCC) 90028) and *C. glabrata* (Refik Saydam Culture Collection (RSKK) 04019) strains were used in this study. The strains were inoculated onto Sabouraud dextrose agar (Acumedia, USA) plates from glycerol stocks and incubated at 35 °C for 24 h. They were then subcultured on the same medium for a further 24 h at 35 °C. The yeast inocula were prepared by diluting the overnight culture with 0.9% NaCl to 1-5 × 10^6 CFU/mL compared to the isolate density standard (Phoenix Spec Nephelometer, Becton Dickinson, USA). The yeast suspensions were then further diluted in RPMI-1640 (with L-glutamine, without sodium bicarbonate) (Sigma, USA) supplemented with 0.165 M morpholinepropanesulfonic acid (MOPS) to a final inoculum ranging between 0.5 × 10^3 and 2.5 × 10^3 CFU/mL.

Antifungal agent: The antifungal agent fluconazole (Mustafa Nevzat, Turkey) was dissolved in distilled water at a final concentration of 2560 mg/L. Ten 2-fold serial dilutions were prepared in RPMI-1640 medium, with final drug concentrations ranging from 64 to 0.125 μg/mL of fluconazole, according to CLSI M27-A2 (3).

Susceptibility testing: Fluconazole MICs were determined using the broth microdilution method described in CLSI M27-A2 for yeasts.

Growth curves: Fungal inocula (100 μL) were added to each well of a sterile, 96-well flat-bottomed microtiter plate containing the test concentration of fluconazole (100 μL/well). Each concentration was tested in duplicate for each organism. Two wells containing fungal suspension with no drug (growth control) and 2 wells containing only media (background control) were included in this plate. ODs were measured for 24 h at 35 °C using a multidetection microplate reader (Bio-Tek-Synergy HT microplate reader, Bio-Tek Instruments, USA) at 405 nm and were automatically recorded every 15 min for each well. Turbidimetric growth curves were obtained depending on the changes in the OD of fungal growth for each drug concentration and the drug-free growth control.

In order to determine fluconazole MICs using the spectrophotometric kinetic assay, the growth percentage for each drug concentration was calculated with the following equation: growth % = [(OD_{405} of wells containing the drug/OD_{405} of the drug-free well) × 100] after the subtraction of background ODs (ODs of microorganism-free wells).

Using Microplate Data Collection and Analysis Software (Bio-Tek Instruments), the highest OD (ODmax), time at mean maximum OD, changes in ODs (delta OD = OD_{final} – $OD_{initial}$) for each well, and inhibition percentage values were calculated. MIC curves and MIC curve interpolations for each organism were obtained using these values.

Results

The growth curves of *C. albicans* and *C. glabrata* during the incubation period in the presence of increasing concentrations of fluconazole were obtained. The growth curves of both strains in each well were monitored every minute from the beginning of the incubation period. With the increase of the fluconazole concentration inside the wells, an inhibition was observed in the growth curves.

Using the microbroth kinetic assay, MIC_{90} values of fluconazole were determined as 0.31 µg/mL for *C. albicans* and 16.32 µg/mL for *C. glabrata* in the first 20 h of the incubation period. Using the broth microdilution method, fluconazole MICs were determined as 0.5 µg/mL for *C. albicans* and 8 µg/mL for *C. glabrata* at 48 h.

MIC curves and MIC curve interpolations for each strain are given in Figures 1 and 2 and the Table, respectively.

Discussion

Early appropriate therapy may alter the course of fungal infections especially, in immunodeficient or immunosuppressed patients. Therefore, early determination of an organism's drug susceptibility may contribute to successful treatment (1,10). Researchers have performed a number of studies on antifungal susceptibility testing in order to provide clinicians with useful susceptibility information sooner (4,8,11).

Ostrosky-Zeichner et al. (11) investigated the correlation of 48- and 24-h fluconazole MICs in 505 *Candida* isolates according to the CLSI M27-A2 method, and they showed good correlation between 48- and 24-h visual MICs. Furthermore, Espinel-Ingroff et al. (4) compared the performance of 24-h visual and 48-h spectrophotometric MIC endpoints to reference the 48-h microdilution broth visual MICs (CLSI M27-A2 document) of fluconazole, itraconazole, voriconazole, and posaconazole for *Candida* spp. They reported that the performance of both investigational MIC endpoint readings gave results similar to those of reference MICs. They emphasized that spectrophotometric MICs are more objective than visual MICs.

Table. MIC curves interpolations of fluconazole against *C. albicans* and *C. glabrata*.

Inhibition %	Concentrations of fluconazole (µg/mL)	
	C. albicans	*C. glabrata*
10	0.027	0.421
20	0.042	0.522
30	0.057	0.659
40	0.072	0.853
50	0.090	1.146
60	0.113	1.628
70	0.144	2.544
80	0.196	4.808
90	0.315	16.328

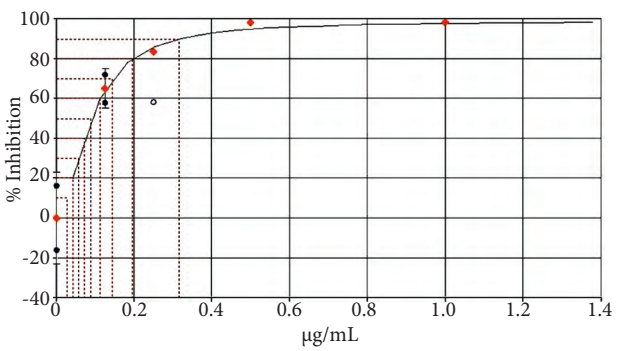

Figure 1. MIC curve of fluconazole against *C. albicans* (Y, inhibition %; X, concentrations of fluconazole).

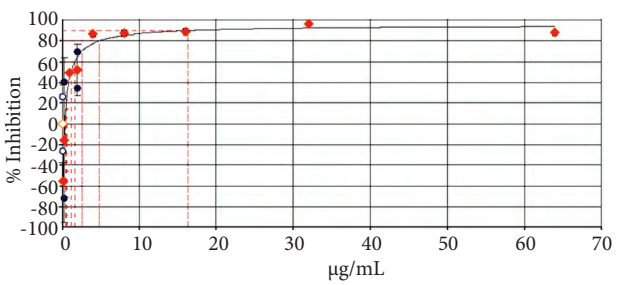

Figure 2. MIC curve of fluconazole against *C. glabrata* (Y, inhibition %; X, concentrations of fluconazole).

In this study, a microbroth kinetic system, which provides kinetic data throughout the incubation period, was used to determine antifungal activity. We were able to observe the effects of fluconazole on both strains by examining turbidimetric growth curves at any desired time-point. We measured the ODs of fungal growth kinetically in each well at selected time intervals and calculated the MICs of the drug using delta ODs in a shorter time than the reference method (approximately 16-20 h) in this assay.

Kinetic measurements of microorganism susceptibilities to antimicrobial agents can also be performed by bioluminescence-, fluorescence-, and OD-based real-time assay using a multidetection microplate reader (9,12). There are only a limited number of studies using this method on fungi, especially yeasts. Balajee et al. (12) used fluorescence-based a microplate assay to determine the antifungal drug susceptibility of *Aspergillus* species. They were able to calculate the MICs of various antifungal drugs against conidia in 16 h with this method by measuring the fluorescence using a viability dye. They found that their results correlated with the reference microdilution method. Meletiadis et al. (8) showed that the antifungal drug resistance of various filamentous fungi could be determined earlier using a microbroth kinetic system based on continuous monitoring of OD fungal growth over a period of time. They determined itraconazole, terbinafine, and amphotericin B resistance using this system in incubation periods of 5.0 to 7.7 h for *Rhizopus oryzae*, 8.8 to 11.4 h for *Aspergillus fumigatus*, 6.7 to 8.5 h for *Aspergillus flavus*, and 13 to 15.6 h for *Scedosporium prolificans* while awaiting formal MIC determination by the CLSI reference method at 24-48 h.

Using kinetic measurements, fungi growth characteristics can be monitored even in the time interval from exposure to visible effect for antifungal agents in real-time. Hence, the effects of antifungals on a target fungus can be observed at any desired time-point of the incubation period, and they may thus be useful for the detection of in vitro antifungal resistance at an earlier stage. The results can also be detected during exposure and are more objective because the MICs can be determined kinetically through spectrophotometric analysis (7,8,12).

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