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ANTIFUNGAL SUSCEPTIBILITY TESTING OF YEASTS WITH BROTH MICRODILUTION AND E-TEST METHODS

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SUMMARY

In vitro susceptibilities were determined for 60 clinical yeast isolates against amphotericin B, fluconazole, and ketoconazole by the broth microdilution technique following NCCLS's Document (M27-A), and a comparative evaluation of the E-test and the broth microdilution methods for antifungal susceptibility testing of 20 clinical yeast isolates against these antifungals was conducted. The susceptible-dose dependent isolates of *Candida albicans* and *Candida krusei* for fluconazole were found. The higher MICs of ketoconazole for some *C.albicans* and *C.krusei* were detected in this study. The correlation between the E-test and the broth microdilution methods were 100 % for amphotericin B, 90 % for ketoconazole and 85 % for fluconazole. E-test is an alternative method for antifungal susceptibility testing of the clinical laboratory, but that further evaluations are needed.

ÖZET

Buyyonda mikrodilüsyon ve E-test yöntemleri ile mayaların antifungal duyarlılığı.

NCCLS'in Document M27-A ile önerdiği mikrodilüsyon yöntemi ile 60 klinik örnekten izole edilen maya suşlarının amfoterisin B, flukonazol, ketokonazole in-vitro duyarlılığının belirlenmesi ve bu antifungallere 20 suşun duyarlılığına göre E-test ve buyyonda mikrodilüsyon yönteminin karşılaştırılması amaçlanmıştır. Flukonazole doza bağlı duyarlı *Candida albicans* ve *Candida krusei* suşları bulunmuştur. Bazı *C.albicans* and *C.krusei* suşları için ketokonazol MIC'larının yükseldiği belirlenmiştir. E-test ve buyyonda mikrodilüsyon yöntemi arasında amfoterisin B'de % 100, flukonazolde % 85, ketokonazolde % 90 uyum belirlenmiştir. Sonuç olarak, E-testin rutin laboratuvarlarda antifungal duyarlılık testi için alternatif yöntem olarak kullanılabileceği belirlenmesine rağmen, bu konuda ek çalışmalara gerek vardır.

INTRODUCTION

The increased incidence of fungal infections, especially yeast infections, led to increased use and efforts for development of antifungal agents. Clinical laboratories are expected to assume a greater role in the selection and monitoring of antifungal chemotherapy (6). As a result of collaborative studies, consensus within the National Committee for Clinical Laboratory Standards, Document Subcommittee on Antifungal Susceptibility Tests has been achieved and a standardized reference method for broth dilution antifungal susceptibility testing of yeasts has been proposed (NCCLS Document M27-P) (4). Macrodilution method has been adapted to a microdilution format with excellent results (5).

This article reports a preliminary evalutaion, in a few species, of one of these alterna-

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tives, the E-test (AB Biodisk, Solna, Sweden) for antifungal agents amphotericin B, fluconazole and ketoconazole.

The E-test consists of a thin, inert, and nonporous plastic strip with a continuous gradient of the antibiotic on one side and a minimum inhibitory concentration (MIC) interpretative scale corresponding to 15 twofold dilution on the other side (B) (1). The E-test is performed by placing the strip on the surface of the inoculated agar plate. After incubation, an ellipse like area of no growth is formed, and the point where the ellipse intersects with the strip is read as the MIC of the antifungal agent. This method can serve as a standardizing point for the development of alternative testing methods more practical for use in the clinical microbiology laboratory (1,9,10).

The propose of the study was to provide additional data by comparing broth microdilution and E-test antifungal susceptibility testing of yeast isolates for amphotericin B, fluconazole and ketoconazole.

MATERIALS AND METHODS

Test organisms: Sixty yeast isolates were tested for their susceptibilities to amphotericin B, fluconazole, and ketoconazole by the broth microdilution method. The yeast isolates included *C.albicans* (n=40), *C.tropicalis* (n=4), *Candida kefyr* (n=4), *Candida krusei* (n=3), *Candida parapsilosis* (n=1), *Candida guilliermondii* (n=1), and *Torulopsis* spp. (n=4), *Geotrichum candidum* (n=3). Nineteen *Candida albicans* and one *Candida tropicalis* were examined for their susceptibilities to amphotericin B, fluconazole, and ketoconazole by E-test method. The majority were from blood or normally sterile body fluids. The isolates were identified by standard methods (11) and stored at -20°C in tryptic soy broth containing 10 % glycerine until used in the study. Prior to use, yeasts were thawed, subcultured at least twice on Sabouraud dextrose agar plates.

Quality control was performed by testing the following strains according to the recommendations of NCCLS Document M27-P (4): *C.albicans* ATCC 90028, *C.albicans* ATCC 64547.

Drugs: Amphotericin B (Sigma Co. St. Louis, USA), fluconazole (Fako Co. Istanbul, Turkey), and ketoconazole (Bilim Co. Istanbul, Turkey) were supplied as powders, and 1280 µg/ml stock solutions of amphotericin B and ketoconazole were prepared by dissolving in dimethyl sulfoxide, and fluconazole in sterile water and stored at -70°C until used. The E-test antifungal gradient strips were purchased from the manufacturer (AB Biodisk, Solna, Sweden) and stored at -20°C until they were used in the study.

Broth microdilution testing was performed according to NCCLS guidelines (5). The turbidity of yeast suspension was adjusted to 0.5 McFarland standard spectrophotometrically by measuring at 530 mm wavelength, and RPMI 1640 medium (with L-glutamine, without bicarbonate) buffered to pH 7.0 with 0.165 M morpholinepropane sulfonic acid (MOPS) buffer (Sigma Co. St. Louis, USA). The wells of their microdilution tray were reconstituted by the addition of the inoculum suspension diluted with RPMI-1640 medium to yield a final inoculum size of 0.5 - 2.5x10^3 cells/ml. Two-fold serial dilution of antifungals were prepared in RPMI-1640 medium to obtain final drug concentrations from 0.03 to 32 µg/ml. The trays were incubated in air at 35°C and were observed for the presence or absence of growth at 48 h. The MIC endpoint was determined according to NCCLS recommendations (complete absence of growth for amphotericin B and 80 % reduction in turbidity for azoles).

The E-test was performed by inoculation of agar plate containing of RPMI-1640 mixed with agar (1.5 %) by using a cell suspension adjusted to a 0.5 McFarland standard. The moisture was allowed to dry for 15 min. and the E-test strips were applied. The plates were incubated at 35°C and were read at 24-48 h, according to the manufacturer's instructions (E-test technical procedures). For azoles, if diffuse growth of microcolonies up to the strip was observed, the MIC endpoint was selected at the point of approximately 80 % growth inhibition and complete absence of growth for amphotericin B.

The E-test and microdilution MIC endpoints of each test condition were considered to agree when the differences between the MIC endpoint pair were within two dilutions (± one dilution).

RESULTS

Table 1 summarizes the in-vitro susceptibilities of 60 yeast isolates to amphotericin B, fluconazole, and ketoconazole as judged by the microdilution method. Amphotericin B was active (MIC for 100 % of isolates tested < 1.0 μg/ml) for all species (except *C.tropicalis*, MIC of 1.0 μg/ml). For fluconazole, in-vitro one susceptible-dose dependent isolate of *C.albicans* and *C.krusei* showed MICs of ≥ 8 μg/ml. Some *C.albicans* and *C.krusei* for ketoconazole showed MICs of ≥ 8 μg/ml.

The MICs for the two control organisms were within a close range for both E-test and microdilution methods (for *C.albicans* ATCC 90028: fluconazole, 0.19 to 0.5 μg/ml; ketoconazole, 0.125 to 0.19 μg/ml; amphotericin B, 0.25 to 0.5 μg/ml) (for *C.albicans* ATCC 64547: fluconazole, 0.5 to 1 μg/ml; ketoconazole, 0.06 to 0.125 μg/ml, amphotericin B, 0.125 to 0.25 μg/ml).

Table 1. The susceptibilities of 60 yeast isolates to amphotericin B, fluconazole, and ketoconazole*.

Organism (no.tested)	Amphotericin B(μg/ml)			Fluconazole (μg/ml)			Ketoconazole (μg/ml)		
	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
C. albicans (n=40)	0.125	0.25	0.03-0.5	0.25	0.5	0.03-16	0.06	1	0.03-4
C. tropicalis (n=4)	0.125	0.25	0.06-1	0.25	0.25	0.125-0.5	0.125	0.125	0.06-0.125
C. kefyr (n=4)	0.125	0.25	0.125-0.25	0.25	0.25	0.03-0.5	0.06	0.06	0.06-0.25
C. krusei (n=3)	0.125	0.25	0.125-0.5	1	1	0.25-16	0.25	0.25	0.06-8
C. parapsilosis (n=1)	-	-	0.125	-	-	0.06	-	-	0.03
C. guilliermondii (n=1)	-	-	0.25	-	-	0.5	-	-	0.06
Torulopsis spp. (n=4)	0.125	0.125	0.125-0.5	0.125	0.125	0.125-0.5	0.06	0.06	0.06-0.25
G. candidum (n=3)	0.03	0.03	0.03-0.5	0.03	0.03	0.03-0.5	0.03	0.03	0.03-0.25

*MICs were determined by the RPMI broth microdilution method. 50% and 90% MICs at which 50 and 90% of isolates were inhibited, respectively.

The range of MICs of three antifungal agents for nineteen *C.albicans* and *C.tropicalis* as well as MIC_{50} and MIC_{90} of isolates were inhibited are summarized in Table 2.

Table 2. Antifungal susceptibility of 20 clinical isolates as determined by the E-test method and the microdilution method.

Organism (no.tested)	Amphotericin B(μg/ml)			Fluconazole (μg/ml)			Ketoconazole (μg/ml)		
	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
C. albicans (n=19)									
E-test	0.125	0.25	0.03-0.5	0.5	1	0.125-64	0.06	4	0.03-64
Microdilution	0.125	0.25	0.03-0.25	0.5	1	0.125-16	0.06	1	0.03-8
C.tropicalis (n=1)									
E-test	-	-	1	-	-	0.125	-	-	0.06
Microdilution	-	-	1	-	-	0.125	-	-	0.06

Table 3 summarizes the percentage agreement of amphotericin B, fluconazole and ketoconazole MICs obtained by the E-test method and the microdilution method. MICs were considered to agree when the differences between the tests were within 2 dilutions (± 1 dilution). Agreement between the E-test method and the microdilution method was good for amphotericin B (100 %), fluconazole (85 %) and ketoconazole (90 %).

Table 3. Percent agreement of the E-test and the microdilution methods.

Organism (no.tested)	% agreement		
	Amphotericin B	Fluconazole	Ketoconazole
C.albicans (n=19)	100	84.2	89.5
C. tropicalis (n=1)	100	100	100
All organisms (n=20)	100	85	90

DISCUSSION

The NCCLS antifungal collaborative study using the broth macrodilution method (7) and the microdilution method (2), evaluated the effect of medium, incubation time (24 versus 48h) and incubation temperature (30 versus 35°C) on intra-and inter-laboratory variations of MIC endpoints of amphotericin B, flucytocine, and ketoconazole. The highest agreement among laboratories, including the rank order of susceptibility, was obtained with RPMI 1640 medium at 35°C and after a 48h incubation time with antifungal compounds. Because of this, we chose the buffered RPMI 1640 medium, the microdilution method, and 48h incubation time for our susceptibility and comparison study.

As a consequence of increasing number of infections caused by yeasts in immunocompromised patients, the use of the antifungal agent has also been more frequent (8). Repeated treatments with azoles have led to the appearance of yeast isolates resistant in-vitro to these agents (3,12, 13) suggest that this picture may change. In this study, amphotericin B was most active (MIC for 100 % of isolates tested ≤ 1.0 μg/ml) against all species (except *C. tropicalis,* MIC of 1.0 μg/ml). The higher MICs of azoles among isolates of *C.albicans* and *C.krusei* were found. The fact that in-vitro susceptibility testing was able to detect these clinically resistant isolates is promising.

The E-test is based on the diffusion of a continuous concentration gradient of drug from a plastic strip into an agar medium. The present study confirmed the results of previous comparisons between the E-test and the broth microdilution methods for amphotericin B, fluconazole, and ketoconazole (1,10). Espinel-Ingroff (1) demonstrated excellent agreement (≥ 90 %) between E-test and the broth macrodilution methods for fluconazole and flucytosine against clinical yeast isolates. Sewell et al. (10) obtained good agreement (94 %) between E-test and the broth dilution methods for the fluconazole susceptibility testing of *Candida* isolates. In contrast, Ruhnke et al. (9) found that the agreement between the microdilution and E-test methods was 67 % for fluconazole, 64 % for ketoconazole, and 78 % for amphotericin B. Although E-test is expensive according to the microdilution methods, this method has the advantage of being simple and easy to use (10). The main problem associated with the agar diffusion E-test was the difficulty in defining the precise borders of the inhibition zone, particularly when azoles tested. In this study, to prevent variability in endpoint reading, plates were always scored by the same person, and combined with the use control species.

It could be concluded that a good agreement is present between the E-test and the microdilution methods for amphotericin B, fluconazole, and ketoconazole antifungal susceptibility testing of yeast isolates. The preliminary data presented here support the continued evaluation of the E-test as an alternative method for amphotericin B and azoles susceptibility testing of yeast isolates, particularly attractive for use in the busy clinical microbiology laboratory.

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