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Comparative evaluation of the antifungal susceptibility of *Candida* isolates from blood specimens: results of a study in a tertiary care hospital in Bursa, Turkey

Canan EVCİ, Beyza ENER, Güher GÖRAL, Sevim AKÇAĞLAR

Aim: Antifungal susceptibility testing may be an important aid in the treatment of patients with life threatening yeast infections. The aim of this study was to determine the susceptibility of *Candida* isolates obtained from clinical specimens to the antifungal agents amphotericin B and fluconazole, which are frequently used in our clinical practice.

Materials and methods: Susceptibility to antifungal agents was investigated using the Etest (AB Biodisk, Solna, Sweden) and the reference broth macrodilution susceptibility test (CLSI M27-A2). The MICs obtained with each of the methods were read after 24 h and 48 h of incubation.

Results: Overall agreement rates within ±1 dilution between the 2 methods were 86.7% (24 h) and 94.1% (48 h) for amphotericin B, and 85.5% (24 h) and 73.3% (48 h) for fluconazole. All *C. albicans, C. pelliculosa, C. glabrata,* and *C. kefyr* isolates were inhibited by ≤1 µg mL−1 of amphotericin B, and fluconazole resistance was not observed in *C. albicans* or *C. tropicalis* isolates according to the 2 methods.

Conclusion: According to the results of this study, decreased susceptibility to fluconazole and amphotericin B was most prominent in *C. parapsilosis, C. krusei,* and *C. glabrata* isolates.

Key words: *Candida* species, antifungal susceptibility tests

Kan örneklerinden izole edilen *Candida* kökenlerinin antifungal duyarlılıklarının karşılaştırmalı değerlendirmesi: üçüncü basamak bir hastane'nin çalışma sonuçları (Bursa-Türkiye)

Amaç: Antifungal duyarlılık testleri, yaşamı tehdit eden maya mantarı enfeksiyonu olan hastaların tedavisinde büyük önem taşımaktadır. Bu çalışmanın amacı klinik örneklerden soyutlanan *Candida* kökenlerinin pratik uygulamada sıklıkla kullanılan antifungal ilaçlar olan amfoterisin B ve flukonazole duyarlılıklarının belirlenmesidir.

Yöntem ve gereç: Antifungal ilaçlara duyarlılık, Etest (AB Biodisk, Solna, İsveç) ve referans broth makrodilüsyon duyarlılık testi (CLSI M27-A2) ile araştırıldı. MİK değerleri, her iki yöntem ile 24 ve 48 saatlik inkübasyonun sonunda değerlendirildi.

Bulgular: İki yöntem arasında (±) 1 dilüsyon içinde uyum oranları, amfoterisin B için % 86,7 (24 saat) ve % 94,1 (48 saat), flukonazol için % 85,5 (24 saat) ve % 73,3 (48 saat) idi. *C. albicans, C. pelliculosa, C. glabrata* ve *C. kefyr* kökenlerinin tümü, ≤1 µg mL−1 konsantrasyonda amfoterisin B ile inhibe oldu ve *C. albicans* ya da *C. tropicalis* kökenlerinde her iki yöntem ile flukonazole direnç saptanmadı.

Sonuç: Bu çalışmada, flukonazol ve amfoterisin B'ye duyarlılığın azalması, en belirgin olarak *C. parapsilosis, C. krusei* ve *C. glabrata* kökenlerinde görüldü.

Anahtar sözcükler: *Candida* türleri, Antifungal duyarlılık testleri

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Uludağ University, Faculty of Medicine, Department of Microbiology, Bursa - TURKEY

Correspondence: *Beyza ENER, Department of Microbiology, Faculty of Medicine, Uludağ University, 16059 Görükle, Bursa - TURKEY*
E-mail: bener@uludag.edu.tr

Introduction

The increasing incidence of fungal infections, the growing number of antifungal agents, the existence of resistant strains, and evidence that supports a correlation between antifungal susceptibility test results and clinical outcome have propelled interest in clinically relevant methods of testing antifungal susceptibility. Unlike antibacterial susceptibility testing, reliable antifungal susceptibility testing has existed for only about 20 years and remains largely in its infancy. Although some unsolved problems and limitations remain, the reference broth dilution method (Clinical and Laboratory Standards Institute: CLSI document M27-A2) is widely used for testing antifungal susceptibility and has supplied data useful to clinical practice; however, antifungal therapy (dose, timing, and administration route), host factors (CD4 cell number and other risk factors), and the pharmacokinetic properties of antifungal drugs are other important factors that determine clinical outcome (1).

Although standardized broth dilution methods for antifungal susceptibility testing are available, easier testing procedures are needed. The Etest (AB Biodisk, Solna, Sweden) was introduced as a means of producing an accurate quantitative MIC result via an agar diffusion format, and several studies have used it successfully for antifungal susceptibility testing of yeasts (2-7).

The present study aimed to determine the in vitro effect of amphotericin B and fluconazole (frequently used antifungal agents in our hospital) against various *Candida* species isolated from blood samples using the reference CLSI broth macrodilution procedure (M27-A2) and Etest, and to compare the results obtained with the 2 methods.

Materials and methods

Candida isolates

The study included 86 *Candida* strains isolated from blood samples during an 18-month period. Each strain represented a unique patient isolate. They were identified to species level according to conventional techniques (germ tube production and morphological features on cornmeal agar with Tween 80) and API ID 32 (bioMerieux, France). In all, 36 strains of *C. albicans,* 15 strains of *C. parapsilosis,* 10 strains of *C. tropicalis,* 9 strains of *C. krusei,* 7 strains of *C. pelliculosa,* 4 strains of *C. glabrata and C. kefyr*, and 1 strain of *C. zeylanoides* were identified. Prior to testing, all isolates were subcultured on Sabouraud dextrose agar (SDA) at least twice to ensure purity and viability. Quality control strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were included as control isolates in all the experiments.

Antifungal drugs

Amphotericin B (Sigma Chemical Co., St. Louis, MO, USA) and fluconazole (Pfizer Co., İstanbul, Turkey) were supplied as reference grade powders. Stock solutions of amphotericin B (1600 µg mL^{-1}) and fluconazole (5200 µg mL^{-1}) were prepared in dimethyl sulfoxide and sterile distilled water, respectively, and were stored at −80 °C until use.

CLSI broth macrodilution method

Broth macrodilution was performed in accordance with CLSI M27-A2 guidelines (8). The minimal inhibitory concentration (MIC) for fluconazole was the lowest concentration at which 80% inhibition of growth occurred relative to that of the drug-free control, and for amphotericin B the MIC was the lowest concentration of the agent that prevented visible growth (8).

Etest method

Etest strips containing a continuous concentration gradient of amphotericin B (0.002-32 µg mL^{-1}) and fluconazole (0.016-256 µg mL^{-1}) were obtained from AB Biodisk (Solna, Sweden). All strips were stored at −20 °C until use. The Etest was performed by inoculating 150-mm petri plates containing 60 mL of RPMI 1640 supplemented with 1.8% agar and 2% glucose with a non-toxic swab using a cell suspension adjusted to a 0.5 McFarland standard. After allowing the agar to absorb the excess moisture, the Etest strips were applied. The plates were incubated at 35 °C and were read after 24 h and 48 h. The MIC for amphotericin B was the lowest concentration on the Etest strip that resulted in 100% inhibition of the organism. The MIC for fluconazole was the lowest concentration that resulted in 80% inhibition of growth, as described and illustrated in the Etest technical guide for antifungal susceptibility testing (9). Because the Etest scale has a continuous

concentration gradient instead of the 2-fold dilutions that the broth macrodilution method test has, the MIC determined with the Etest was raised to the next 2-fold dilution level of the reference method for the sake of comparison.

Analysis of the results

All Etest MICs (read after 24 h and 48 h) were compared to the CLSI reference macrodilution MICs (read after 24 h and 48 h). The data included both on-scale and off-scale results. High off-scale MICs were converted to the next higher concentration and low off-scale results were unchanged. Differences in MIC endpoints ≤1 dilution (±1 dilution) were used to calculate the percentage of agreement.

CLSI breakpoints were used for fluconazole. Isolates were considered susceptible if their MIC was ≤8 µg mL^{-1}, susceptible-dose dependent (S-DD) if their MIC was 16-32 µg mL^{-1}, and resistant if their MIC was ≥64 µg mL^{-1} (8). Although interpretive criteria have not yet been defined for amphotericin B, for comparison purposes the present study considered an isolate susceptible if its MIC was ≤1 µg mL^{-1} (10-15).

Results

MICs for the 2 control organisms tested with the reference method in all the experiments consistently agreed with the CLSI reference results, confirming both the reproducibility of the results and that the drug concentrations were properly prepared. Control organism MICs obtained with the Etest were also in the range previously reported.

Table 1 summarizes the 86 *Candida* strains' susceptibility to amphotericin B, and the percentage of agreement between the 2 methods after 24 h and 48 h of incubation. Because of insufficient growth after 24 h, data were not obtained for 1 strain of *C. zeylanoides*

Table 1. Amphotericin B susceptibility results according to broth macrodilution (MD) and the Etest, and agreement rates between the 2 methods.

Species (Number)	Method	MIC (µg mL^{-1}) 24 h			MIC (µg mL^{-1}) 48 h			Agreement (%)	
		MIC range	**MIC_{50}**	**MIC_{90}**	**MIC range**	**MIC_{50}**	**MIC_{90}**	24 h	48 h
C. albicans (36)	MD	0.125-0.5	0.25	0.5	0.25-1	0.5	0.5	83.3	100
	Etest	0.064-0.25	0.125	0.25	0.25-1	0.5	0.5		
C. parapsilosis (15)	MD	0.25-0.5	0.25	0.5	0.5-2	0.5	1	92.3	92.9
	Etest	0.125-0.5	0.125	0.5	0.25-2	0.5	1		
C. tropicalis (10)	MD	0.25-0.5	0.25	0.5	0.5-2	0.5	0.5	100	100
	Etest	0.25-0.5	0.25	0.5	0.5-1	0.5	1		
C. krusei (9)	MD	0.5-2	1	1	1-2	1	2	77.8	88.9
	Etest	1-4	1	2	1-4	2	2		
C. pelliculosa (7)	MD	0.125-0.5	0.25	0.5	0.25-1	0.5	1	71.4	57.1
	Etest	0.032-1	0.125	0.5	0.032-1	0.25	0.5		
C. glabrata (4)	MD	0.25-0.5	*	*	0.5-1	*	*	100	100
	Etest	0.5			0.5-1				
C. kefyr (4)	MD	0.5	*	*	0.5-1	*	*	100	100
	Etest	0.5-1			0.5-1				
C. zeylanoides (1)	MD	1	*	*	2	*	*		100
	Etest	**			4				
Total (86)	**MD**	**0.25-2**	**0.25**	**0.5**	**0.25-2**	**0.5**	**1**	**86.7**	**94.1**
	Etest	**0.032-4**	**0.25**	**1**	**0.032-4**	**0.5**	**2**		

*The number of strains was insufficient for assessment. **One strain exhibited insufficient growth.
MIC_{50}: The concentration of amphotericin B necessary to inhibit 50% (MIC_{50}) of the isolates tested.
MIC_{90}: The concentration of amphotericin B necessary to inhibit 90% (MIC_{90}) of the isolates tested.

(Etest) and 1 strain of *C. parapsilosis* (broth macrodilution). Data for these isolates were not included in the final evaluation of values. After 48 h of incubation the Etest and broth macrodilution MIC_{50} and MIC_{90} values were generally 2-4-fold greater than those obtained after 24 h. Although MIC_{50} and MIC_{90} values obtained with the 2 methods were similar, the MIC range based on the Etest was wider than that obtained with the broth macrodilution method. There was better agreement between the 2 methods' MIC values after 48 h of incubation than after 24 h, and the MICs for *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. kefyr*, and *C. zeylanoides* were the same. Overall agreement between the 2 methods was very acceptable: 94.1% after 48 h of incubation.

Although interpretive criteria have not yet been defined for amphotericin B, most studies have used ≤1 $\mu g\ mL^{-1}$ as a breakpoint value for this drug. After 48 h of incubation 93% of isolates had an MIC value ≤1 $\mu g\ mL^{-1}$ based on broth macrodilution, versus 89.5% based on the Etest. Amphotericin B was activity was maximal against *C. albicans*, *C. pelliculosa*, *C. glabrata*, and *C. kefyr* isolates (all MICs ≤1 $\mu g\ mL^{-1}$) according to both test methods, followed by *C. tropicalis* and *C. parapsilosis* based on broth macrodilution after 48 h (≥90%). Amphotericin B was least active against *C. krusei* isolates according to both test methods (Table 2).

Table 3 shows the MIC range of fluconazole for the 86 isolates tested with both methods; MICs for several isolates were high. Because of insufficient growth after 24 h data were not obtained for 1 strain of *C. albicans* (Etest), 1 strain of *C. kefyr* (Etest), and 1 strain of *C. zeylanoides* (broth macrodilution). Data for these isolates were not included in the final evaluation of values.

After 48 h of incubation the MIC_{50} and MIC_{90} values of fluconazole were generally 2-4-fold greater than the values obtained after 24 h. A large increase in the MIC_{90} at 48 h occurred for *C. albicans* and *C. tropicalis* based on the broth macrodilution method, probably due to the trailing endpoint effect (Table 3). The MICs for 5 *C. albicans* (5/36 = 13.9%) and 5 *C. tropicalis* (5/19 = 50%) isolates were >64 $\mu g\ mL^{-1}$ according to the reference method, but only 0.25-0.50 $\mu g\ mL^{-1}$ based on the Etest. It is important to note that these 10 isolates demonstrated very clear MIC endpoint readings based on the Etest, but heavy trailing according to the broth macrodilution test. Thus, if the trailing effect is ignored, none of the *C. albicans* or *C. tropicalis* isolates was observed to be fluconazole resistant at either incubation time.

Dose-dependent susceptibility or resistance was observed in isolates other than those of *C. albicans* and *C. tropicalis* in the present study (Table 4). Decreased susceptibility was seen in 5 of the *C. parapsilosis* isolates that were S-DD according to the reference test, and all of them were resistant according to the Etest after 24 h of incubation. After 48 h of incubation 2 of these 5 S-DD strains also became resistant according to the broth macrodilution method. All intrinsically fluconazole-resistant *C. krusei* isolates had high MIC values at both temperatures according to both test methods, and resistance or S-DD was observed in all *C. glabrata* isolates after 48 h of incubation. *Candida pelliculosa* is considered a rare pathogen and 7 of its isolates had higher MIC values that *C. albicans* and *C. tropicalis*. One of the *Candida pelliculosa* isolates was S-DD according to the reference method, but was resistant according to the Etest at both temperatures. One of the *C. kefyr* isolates was S-DD after 48 h of incubation according to the reference method, but remained susceptible according to the Etest, which was considered a non-comparable result. One *C. zeylanoides* isolate was resistant according to both test methods.

The overall agreement for fluconazole between the 2 test methods was lower than that for amphotericin B, probably due to the trailing endpoint effect observed with azoles after 48 h of incubation, especially for *C. albicans* and *C. tropicalis* (Table 3). Among the disagreements, 10 were due to the trailing effect; if this effect is ignored, higher agreement can be obtained between the 2 test methods.

Discussion

The present study used the CLSI reference broth macrodilution (M27-A2) and Etest methods to determine the susceptibility of 86 *Candida* isolates to amphotericin B and fluconazole. Several studies have evaluated the Etest for assessing antifungal susceptibility (2-7). These studies have demonstrated

Table 2. Percentage of isolates with MIC values ≤1 μg mL^{-1} for amphotericin B.

Strains	**Method**	**Incubation time (h)**	**%**	
			MIC ≤ 1 μg mL^{-1}	**MIC ≥ 2 μg mL^{-1}**
C. albicans (36)	MD	24	100	-
		48	100	-
	Etest	24	100	-
		48	100	-
C. parapsilosis (15)	MD	24	100	-
		48	93.3	6.7
	Etest	24	100	-
		48	93.3	6.7
C. tropicalis (10)	MD	24	100	-
		48	90	10
	Etest	24	100	-
		48	100	-
C. krusei (9)	MD	24	88.9	11.1
		48	66.7	33.3
	Etest	24	66.7	33.3
		48	22.2	77.8
C. pelliculosa (7)	MD	24	100	-
		48	100	-
	Etest	24	100	-
		48	100	-
C. glabrata (4)	MD	24	100	-
		48	100	-
	Etest	24	100	-
		48	100	-
C. kefyr (4)	MD	24	100	-
		48	100	-
	Etest	24	100	-
		48	100	-
C. zeylanoides (1)	MD	24	100	-
		48	-	100
	Etest	24		
		48	-	100
Total (86)	**MD**	**24**	**98.8**	**1.2**
		48	**93**	**7**
	Etest	**24**	**96.5**	**3.5**
		48	**89.5**	**10.5**

MD: Broth macrodilution.

Table 3. Fluconazole susceptibility results obtained with broth macrodilution (MD) and the Etest, and agreement rates between the 2 methods.

Species (Number)	Method	**MIC (μg mL^{-1})**						**Agreement** (%)	
		24 h			**48 h**				
		MIC range	**MIC_{50}**	**MIC_{90}**	**MIC range**	**MIC_{50}**	**MIC_{90}**	24 h	48 h
C. albicans (36)	MD	≤0.125-1	0.25	0.5	≤0.125-64	0.25	>64	100	75
	Etest	0.125-1	0.5	0.5	0.25-2	0.5	1		
C. parapsilosis (15)	MD	0.25-32	1	32	0.5->64	2	>64	73.3	73.3
	Etest	0.25->256	1	>256	0.25->64	2	>256		
C. tropicalis (10)	MD	0.25-1	0.5	1	1->64	2	>64	100	50
	Etest	0.5-2	1	2	1-2	2	2		
C. krusei (9)	MD	16-32	32	32	32->64	>64	>64	33.3	66.7
	Etest	64->256		64	>256	>256	>256		
C. pelliculosa (7)	MD	0.5-16	2	4	1-32	8	8	85.7	85.7
	Etest	0.25->256	2	8	0.5->256	8	8		
C. glabrata (4)	MD	4-16	*	*	15->64	*	*	75	100
	Etest	0.5-32			8->256				
C. kefyr (4)	MD	0.25-8	*	*	0.25-32	*	*	100	75
	Etest	0.5-1			0.25-1				
C. zeylanoides (1)	MD	**	*	*	>64	*	*		100
	Etest	>256			>256				
Total (86)	**MD**	**≤0.125-32**	**0.5**	**32**	**≤0.125->64**	**2**	**>64**	**85.5**	**73.3**
	Etest	**0.125->256**			**0.25->256**				

*The number of strains was insufficient for assessment. **One strain exhibited insufficient growth.

MIC_{50}: The concentration of fluconazole necessary to inhibit 50% (MIC_{50}) of the isolates tested.

MIC_{90}: The concentration of fluconazole necessary to inhibit 90% (MIC_{90}) of the isolates tested.

Table 4. *Candida* strains that were susceptible (dose-dependent) (S-DD) and resistant (R) to fluconazole.

Candida species (86)	24 h				48 h			
	MD		Etest		MD		Etest	
	S-DD	R	S-DD	R	S-DD	R	S-DD	R
C. parapsilosis (15)	5	-	-	5	3	2	-	5
C. krusei (9)	9	-	-	9	3	6	-	9
C. pelliculosa (7)	1	-	-	1	1	-	-	1
C. glabrata (4)	1	-	1	-	3	1	2	1
C. kefyr (4)	-	-	-	-	1	-	-	-
C. zeylanoides (1)	-	-	-	1	-	1	-	1
Total (%)	**16 (18.6)**	-	**1 (1.2)**	**16 (18.6)**	**11 (12.8)**	**10 (11.6)**	**2 (2.3)**	**17 (19.8)**

MD: Broth macrodilution.

that this method's results agree very well with reference broth dilution methods. The data obtained in the present study also confirm that there is a good correlation between MICs obtained with the Etest and broth dilution methods. Agreement between the 2 methods for amphotericin B and fluconazole was 94% and 73%, respectively, after 48 h of incubation.

The ability to determine MIC results within 24 h is potentially advantageous for early clinical application of antifungal susceptibility test results. The present study also performed 24-h assessments for both methods, and only a few isolates required 48 h of incubation for optimal growth. The determination of the endpoints for fluconazole is a significant factor in the variability of the MIC results. The usual partial growth inhibition (trailing) observed with this drug makes it difficult to accurately determine MICs (16). Certain yeast isolates (especially *C. albicans* and *C. tropicalis*) produce trailing endpoints, usually after 48 h, with growth evident at all concentrations of the drugs. Isolates are considered to have trailing endpoints according to CLSI testing if their 24-h MICs are <8 μg mL^{-1} (which indicates susceptibility) and if their 48-h MICs are ≥64 μg mL^{-1} (which indicates resistance). This may be due to the azoles' fungicidal activity, which allows several generations of growth before significant inhibition occurs (17). The present study observed trailing endpoints for 14% and 50% of *C. albicans* and *C. tropicalis* isolates, respectively, after 48 h of incubation. The incidence of trailing endpoints is unknown, but appears to be a frequent problem in antifungal susceptibility testing. Based on the literature, the trailing endpoint does not appear to indicate resistance (17,18); thus, CLSI results obtained after 24 h of incubation may be more appropriate for yeast isolates in which trailing endpoints are observed than for isolates in which no such trailing endpoints are observed. In the present study there was also good agreement between the 2 test methods for fluconazole after 24 h of incubation. Moreover, for isolates with MICs ≥64 μg mL^{-1} after 24 h and 48 h, alternative testing methods can be used before considering them resistant (17).

Amphotericin B has broad spectrum fungicidal activity, making it useful as an empirical treatment for suspected fungal infections in critically ill patients. The resistance of *Candida* species to amphotericin B is considered uncommon, but has been documented, especially in *C. lusitaniae* (19,20). Unfortunately, the CLSI method generates a restricted range of amphotericin B MICs, precluding reliable discrimination between susceptible and resistant *Candida* species isolates, and preventing the development of interpretive MIC breakpoints for in vitro testing (21,22). Later, it was shown that broth microdilution MICs and minimal fungicidal concentrations (MFCs) obtained from tests using AM3 medium, as well as Etest MICs, span a broad range of values. (22-25). We also observed that the CLSI broth macrodilution reference method using RPMI 1640 medium produced a restricted range of MIC results for amphotericin B in the present study, but we must keep in mind that important methodological issues still need to be resolved in detecting amphotericin B resistance. Recently, the Etest has been shown to produce the widest distribution of MICs, but none of the test formats has generated results that significantly correlate with therapeutic success or failure (26).

In the present study, according to the 2 test methods amphotericin B was most effective against *C. albicans*, *C. pelliculosa*, *C. glabrata*, and *C. kefyr* isolates (all MICs ≤1 μg mL^{-1}); however, after 48 h of incubation some *C. krusei*, *C. parapsilosis*, *C. tropicalis* isolate MICs were ≥2 μg mL^{-1}. As mentioned, the importance of these isolates with high MIC values is unknown, but some reports emphasize their importance (27-29). Goldman et al. reported that patients treated with amphotericin B at doses >1 mg kg^{-1} of body weight day^{-1} respond significantly better to *C. krusei* infections than other patients do (28). Nevertheless, interpretive breakpoints for amphotericin B susceptibility testing have remained controversial due to conflicting results reported by correlation studies. Future prospective studies using different methods may help to establish guidelines for detecting amphotericin B resistance in *Candida* species.

After 48 h of incubation in the present study fluconazole was active (≤8 μg mL^{-1}) against 75.6% and 77.9% of isolates according to the broth macrodilution and Etest, respectively. If intrinsically resistant *C. krusei* isolates are excluded, fluconazole activity rises to 86% and 88.4% of the isolates, respectively,

according to both test methods. None of the *C. albicans* or *C. tropicalis* isolates exhibited in vitro resistance to fluconazole, confirming the rarity of fluconazole resistance among *C. albicans* isolates outside of AIDS patients with recurrent oropharyngeal candidiasis (1,10). According to population-based and sentinel surveillance studies, the resistance of *C. glabrata* and *C. parapsilosis* to fluconazole has been noted in approximately 10% and 1% of bloodstream infection isolates, respectively, with the exception of high rates (40% and 15%) from Sweden (30). The present study showed that all the *C. glabrata* isolates and 5 *C. parapsilosis* isolates were S-DD or resistant, but we think that the number of isolates was insufficient to make an accurate assessment. Further evaluation of most of our isolates will provide a more accurate susceptibility pattern.

The present study's additional data also confirmed that the Etest has potential utility as an easy alternative method to the NCCLS broth macrodilution method. We emphasize that whichever technique is used, experience determining MICs and careful attention to procedural details are critically important when performing susceptibility tests. Although the number of *Candida* species and antifungal agents used in the present study were limited, the results provide a preliminary idea about the isolates' susceptibility patterns. Further evaluation is needed to assess certain susceptibility patterns and trends in yeast isolates from bloodstream infections. According to the present study, decreased susceptibility to fluconazole and amphotericin B was most prominent among *C. parapsilosis*, *C. krusei*, and *C. glabrata* isolates. Additional research is essential for accurate assessment.

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