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Original Article

Determination of amphotericin B antifungal susceptibility in Candida strains using an inverted microscope

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Abstract

Introduction: Invasive *Candida* infections have recently shown a significant increase in prevalence and are associated with high mortality rates. Initiating early antifungal treatment in patients with candidemia is vital. The aim of our study was to compare the antifungal susceptibility results of a new method called Flat Plate Method modified from reference "Clinical and Laboratory Standards Institute (CLSI) microdilution method by us with Sensititre Yeast One colorimetric method and the reference CLSI method.

Methodology: We tested 100 *Candida* isolates from blood cultures. We followed the CLSI M27-A3 (reference method for broth dilution antifungal susceptibility testing of yeasts; third edition) guidelines for testing *in vitro* susceptibility to amphotericin B. In the Flat Plate method, 96-well plates were used for evaluation with an inverted microscope. Minimum inhibitory concentration (MIC) values in the SYO method were measured following the manufacturer's instructions. The MIC values obtained by all three methods were considered compatible if they were within ± 2 dilution limits.

Results: The SYO method detected *C. albicans* and *C. glabrata* with 100% essential agreement, whereas there was 96.29% essential agreement in the case of *C. parapsilosis*. In the Flat Plate method, the essential agreement with amphotericin B was 91.42%, for *C. albicans* isolates and 89.47%.for *C. glabrata* strains.

Conclusions: When determining early antifungal susceptibility using the Flat Plate method, the results are obtained quickly, with high accuracy, and without incurring additional costs. However, there is a need for comprehensive studies comparing different antifungals.

Key words: *Candida*, amphotericin B, susceptibility.

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Introduction

Invasive *Candida* infections affect an estimated 250,000 patients per year globally and are associated with high morbidity and mortality in immunocompromised patients, particularly in intensive care units [1,2].

Delayed antifungal treatment in invasive candidiasis leads to a twofold increase in mortality every day; therefore, the rapid and accurate detection of antimicrobial resistance is critical [3,4]. Measuring fungal growth in the presence of different antifungal drug concentrations allows the determination of the minimal inhibitory concentration (MIC), a value that helps predict the likely efficacy of the antifungal therapy [3]. Microdilution methods are the gold standard for reference techniques. Two organizations, the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI), have standardized methods to perform antifungal susceptibility testing [4,5]. Despite the differences between these two methods, their results have been demonstrated to be comparable and are used worldwide [6]. However, one of the limitations when using reference methods or commercial adaptations of microdilution (e.g., Etest® [AB Biodisk, Solna, Sweden] or Sensititre™ Yeast One [SYO, Thermo Fisher Scientific, MA, USA]) is that these methodologies have a time-consuming set up and intrinsically slow turnaround times [7-10]. In response to the uncertainties of antifungal susceptibility testing reference methods, new methods have been developed to determine antifungal resistance in less time [11]. Molecular techniques, flow cytometry, and alternative methods based on matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) are examples of these methods [12,13]. However, these techniques have limited applications in routine laboratories due to the need for more standardization and interpretive guidelines, as well as their increased costs and the need for special equipment and qualified personnel [13,14].

Table 1. Reference method and Flat Plate method conditions for antifungal susceptibility testing.

Characteristic	Reference method	Flat Plate method
Format	Microdilution	Microdilution
Well shape (bottom)	U bottom	Flat
Media	RPMI 1640	RPMI 1640
Glucose content	0.2%	2%
Inoculum size	0.5×10^3 - 2.5×10^3 cells/mL	0.5×10^5 - 2.5×10^5 cells/mL
Incubation temperature	35 °C	35 °C
Incubation time for AMb	24 h	8 h
Endpoint	No growth	No growth

Microbial growth in the presence of an antimicrobial agent is the ultimate indicator of *in vitro* resistance, and visible appearance of growth takes time [15]. Various observation-based methods have been developed to detect this process. The disk diffusion method is such a method [14]. Several studies have shown promising results, indicating that the incubation time for the disk diffusion test can be shortened with no significant impact on sensitivity [16,17]. This study aimed to modify the existing reference CLSI method in *Candida* isolates to create a new method called the Flat Plate method and then to compare the antifungal susceptibility results of this method with the SYO method and the CLSI reference microdilution method.

Methodology

This study included 100 *Candida* isolates obtained from blood culture samples. These strains were identified by germ tube formation, micro-morphology on cornmeal-tween 80 medium, and VITEK-MS MALDI-TOF (bioMérieux SA, Marcy l' Étoile, France). Additionally, *Candida parapsilosis* ATCC® 22019™ and *Candida krusei* ATCC® 6258™ strains were used for quality control. The isolates, stored at -80 °C before the study, were passaged twice onto Sabouraud Dextrose Agar (SDA-Neogen, Lansing, MI, USA) and included after ensuring that a pure culture was obtained.

The *in vitro* susceptibility of strains to amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) was determined using the broth microdilution method recommended in the CLSI M27-A3 guidelines [4]. According to CLSI recommendations, the antifungal drugs were diluted in RPMI 1640 (Roswell Park Memorial Institute; Sigma-Aldrich, St. Louis, MO, USA) medium containing 0.2% glucose to final concentrations ranging from 16 to 0.03 µg/mL in U-bottom plates. The prepared plates were stored at -20 °C until used.

The final inoculum concentration for the plates was prepared to be 0.5×10^3 - 2.5×10^3 cells/mL. The inoculated plates were incubated at 35 °C for 24 hours. At the end of the incubation period, the well in which growth was completely inhibited compared to the control well was determined as the MIC [4].

In the Flat Plate method, unlike the description in the CLSI M27-A3 guidelines, 96-well flat-bottom plates were used to enable evaluation under an inverted microscope (OLYMPUS, Tokyo, Japan). Additionally, optimization studies determined the inoculum to be 0.5×10^5 - 2.5×10^5 cells/mL, and the incubation time for evaluation was eight hours. Two observers conducted evaluations independently, and tests with inconsistent results were repeated. Reference method and Flat Plate method conditions are provided in Table 1.

SYO (Trek Diagnostic Systems, Cleveland, OH, USA) plates were prepared according to the manufacturer's recommendations. The plates were sealed with a strip and incubated at 35 °C for 24 hours. In this method, the range of 0.12-8 µg/mL was evaluated for amphotericin B. The evaluation was done by visual inspection of color changes. A pink color indicated growth, a purple color indicated partial inhibition and a blue color indicated no growth. The first well with no growth inhibition was considered the MIC.

The essential agreement rates of both tests compared to the reference method were calculated according to the US Food and Drug Administration (FDA) guidelines (US FDA. Class II Special Controls Guidance Document) [18].

Table 2. MIC50 and MIC90 values and MIC ranges determined by three different methods.

Antifungals methods	MIC50 (µg/mL)	MIC90 (µg/mL)	MIC range (µg/mL)
Reference method	0.25	1	0.125-1
Sensititre Yeast One	0.5	1	0.125-1
Flat plate method	0.5	0.5	0.125-1

MIC: Minimum Inhibitory Concentration.

Table 3. MIC50, MIC90 values, and MIC ranges obtained using three different methods.

Antifungals Methods	MIC50 (µg/mL)	MIC90 (µg/mL)	MIC Ranges (µg/mL)
C. albicans			
Reference method	0.25	0.5	0.125-1
Sensititre Yeast One	0.25	0.5	≤ 0.12-1
Flat Plate method	0.25	0.5	0.125-1
C. parapsilosis			0.125-1
Reference method	0.5	0.5	0.125-1
Sensititre Yeast One	0.25	0.5	≤ 0.12-1
Flat Plate method	0.25	0.5	0.125-1
C. glabrata			0.125-1
Reference method	0.5	1	0.125-1
Sensititre Yeast One	0.5	1	≤ 0.12-1
Flat Plate method	0.25	0.5	0.125-1
Other*			0.125-1
Reference method	0.5	1	0.125-1
Sensititre Yeast One	1	1	≤ 0.12-1

*Other: *C. krusei:* 6*; C. kefyr:* 5; *C. lusitaniae*: 4; *C. tropicalis:* 2; *C. metapsilosis:* 1; *C. gulliermondii*: 1; MIC: Minimum Inhibitory Concentration.

Results

According to the species identification results of the 100 *Candida* isolates obtained from the blood cultures, 35 (35%) were identified as *Candida albicans*, while 55 (55%) were categorized as non-albicans *Candida* species. Among the non-albicans *Candida* species, there were 27 *Candida parapsilosis*, 19 *Candida glabrata*, 6 *Candida krusei*, 5 *Candida kefyr*, 4 *Candida lusitaniae*, 2 *Candida trcopicalis*, 1 *Candida metapsilosis*, and 1 *Candida guilliermondii*.

Table 2 presents the minimum inhibitory concentration for 50% of the isolates (MIC50) and minimum inhibitory concentration for 90% of the isolates (MIC90) values. The MIC ranges for amphotericin B against the *Candida* isolates analyzed in the study were determined by using three different antifungal susceptibility assays.

At the species level, the MIC ranges obtained with all three methods were 0.125-1 µg/mL. In the case of *C. albicans*, the MIC50 and MIC90 values obtained with all three methods were determined to be 0.25 and 0.5 µg/mL. In the case of *C. parapsilosis* isolates, the MIC50 value (0.5 µg/mL) in the CLSI reference method was higher than in the other two methods. while in the *C. glabrata* strains, the MIC50 and MIC90 values (0.25 and 0.5 µg/mL, respectively) were found to be lower when obtained by the Flat Plate method compared to those obtained by the CLSI and SYO methods. Table 3 presents the MIC50 and MIC90 values and the MIC ranges obtained with the three different antifungal susceptibility tests against the *Candida* isolates included in the study at the species level.

The study observed essential agreement rates for amphotericin B among the tested *Candida* species, including *C. albicans*, *C. parapsilosis*, *C. glabrata*, and other isolates, when subjected to the SYO method, with rates of 100%, 96.29%, 100%, and 89.47%, respectively.

Additionally, for *C. albicans*, *C. parapsilosis*, *C. glabrata*, and other isolates, the essential agreement rates in the Flat Plate method were determined to be 91.42%, 96.29%, 89.47%, and 94.73%, respectively. The essential agreement rates with the reference method for *Candida* species are presented in Table 4.

The isolates of *C. albicans* and *C. glabrata* studied with the SYO method were all in agreement with the reference method, whereas for *C. parapsilosis*, one isolate was found to be two dilutions lower, and for the other isolates, two isolates were two dilutions higher. The results of both methods for all isolates are presented in Table 5.

Discussion

In invasive mycoses, early antifungal treatment is critical for patient survival [19]. Inadequate antifungal treatment or delays in starting adequate treatment increase mortality rates in patients with candidemia [20]. Antifungal susceptibility tests are becoming essential due to the increase in antifungal drug resistance [21]. Since the commonly used methods of

Table 4. Essential agreement rates of Sensititre Yeast One and Flat Plate methods compared to the reference method.

	Sensititre Yeast One				Flat Plate method			
	C. albicans	***C. parapsilosis***	***C. glabrata***	**Diğer**	***C. albicans***	***C. parapsilosis***	***C. glabrata***	**Other***
EU (%)	100	96.29	100	89.47	91.42	96.29	89.47	94.73

*Other: *C. krusei: 6; C. kefyr:* 5; *C. lusitaniae*: 4; *C. tropicalis:* 2; *C. metapsilosis:* 1; *C. gulliermondii*: 1.

Table 5. Agreement rates of Sensititre Yeast One and Flat Plate methods compared to the reference method.

	-3	-2	-1	0	+1	+2	+3
Sensititre Yeast One							
C. albicans	-	-	7	17	11	-	-
(n = 35)			(20%)	(48.57%)	(31.43%)		
C.parapsilosis	-	1	7	13	6	-	-
(n = 27)		(3.70%)	(25.93%)	(48.15%)	(22.22%)		
C. glabrata	-	-	3	8	8	-	-
(n = 19)			(15.80%)	(42.10%)	(42.10%)		
Other	-	-	-	8	9	2	-
(n = 19)				(42.11%)	(47.37%)	(10.52%)	
Flat Plate method							
C. albicans	-	3	6	14	12	-	-
(n = 35)		(8.57%)	(17.14%)	(40%)	(34.28%)		
C. parapsilosis	-	1	4	15	7	-	-
(n = 27)		(3.7%)	(16.66%)	(55.55%)	(25.93%)		
C. glabrata	-	2	3	12	2	-	-
(n = 19)		(10.52%)	(15.79%)	(63.15%)	(10.53%)		
Other	-	1	4	10	4	-	-
(n = 19)		(5.26%)	(21.05%)	(52.63%)	(21.05%)		

MIC: Minimum Inhibitory Concentration; -3, -2, -1: alternative method MIC result is, respectively, three, two and one dilutions lower than the standard method. 0: alternative method MIC result is equal to the standard method. +1, +2, +3: alternative method MIC result is, respectively, one, two and three dilutions higher than the standard method.

antifungal susceptibility testing have a yield time of 24-48 hours, new rapid methods for detecting resistance are required to ensure rapid and adequate adaptation in cases where antifungal treatment is required [22].

The SYO method is an easy-to-implement method for routine laboratory antifungal susceptibility testing based on the CLSI reference method [23]. The CLSI method is widely used in clinical and research laboratories as it provides consistent results in accuracy and reproducibility compared to the reference method [9]. Altınbaş *et al.* compared the results of the SYO method with the CLSI microdilution method using different antifungals in 129 *Candida* isolates [24]. They reported that the basic concordance rates of amphotericin B for *C. albicans*, *C. parapsilosis* and, *C. glabrata* were 100%, 100%, and 87%, respectively. Consequently, the essential compatibility in *Candida* isolates was 99% [24]. The SYO method is an alternate antifungal susceptibility approach that is simple to use and compatible with the CLSI method in clinical laboratories. Cuenca-Estrella *et al*. tested amphotericin B susceptibility in *Candida* spp isolates using the SYO and CLSI broth microdilution methods and determined the sensitivity to be 97.4% [25].

In the present study, the SYO method was compared with the CLSI method, and the essential agreement rates for *C. albicans*, *C. parapsilosis*, *C. glabrata* and other isolates were determined to be 100%, 96.29%, 100%, and 89.47%, respectively. The essential agreement rate for all *Candida* isolates included in the study was 97%. These rates agree with the findings of research published in literature.

Antifungal susceptibility testing is still an essential but challenging tool in the fight against fungal diseases despite there being many factors beyond microbiological resistance that affect *in vivo* outcomes in opportunistic invasive mycoses [26]. Detection of fungal growth in the presence of different concentrations of antifungal drugs helps estimate the effectiveness of the antifungal treatment [27]. In the present study, the CLSI reference method was modified, and the early detection of amphotericin B sensitivity in *Candida* was achieved using an inverted microscope. The results showed essential agreement rates with the CLSI method, and in *C. albicans*, *C. parapsilosis*, *C. glabrata* and other isolates, these rates were 91.42%, 96.29%, 89.47%, and 94.73%, respectively.

In the present study, the MIC50 and MIC90 values for amphotericin B in *C. albicans* isolates were determined to be 0.25 and 0.5 µg/mL, respectively, with all three methods. The MIC ranges were found to be between 0.125 and 1 µg/mL for all three methods. Arıkan *et al*. determined that MIC50 and MIC90 values for amphotericin B in *C. albicans* isolates were 1 and 2 µg/mL, respectively [28]. In the same study, the MIC50 and MIC90 values of amphotericin B in *C. parapsilosis* isolates were determined to be 1 and 2 µg/mL, respectively, while the MIC range was 0.125 and 2 µg/mL. In the present study, MIC50 and MIC 90 values for amphotericin B in *C. parapsilosis* isolates were found to be 0.5 and 0.5 µg/mL, respectively, using the CLSI microdilution method, and they were 0.25 and 0.5 µg/mL, respectively using the SYO and Flat Plate

method. The MIC range was determined to be 0.125-1 µg/mL for all three methods. In this study, the MIC50 and MIC90 values in *C. glabrata* strains were found to be 1 and 2 µg/mL, respectively, while the MIC range was 0.25-2 µg/mL [28]. In present study, the MIC50 and MIC90 values were found to be 0.5 and 1 µg/mL, respectively, in the CLSI and SYO methods, while they were 0.25 and 0.5 µg/mL in the Flat Plate method. In the present study, the results obtained with all three methods were found to be at least one dilution lower for all *Candida* species.

The eighth hour results for the Flat Plate method have a high essential agreement compared to the CLSI reference method. The results can be evaluated visually after 24 hours in the CLSI method; however, an inverted microscope is needed to evaluate the results in the Flat Plate method. Additionally, experienced personnel may be needed to evaluate the Flat Plate method. However, it is critical to determine antifungal sensitivity on the same day in patients with invasive candidiasis.

Although the results obtained are compatible with the reference method, this study's limitations include the absence of resistant isolates and the limited use of antifungals. To clarify the applicability of the Flat Plate method, comparative studies involving many microorganisms and different evaluators are needed.

Conclusions

The SYO and Flat Plate methods showed high agreement compared to the standard method for determining amphotericin B resistance in the *Candida* isolates studied. In addition, these results were obtained in the eighth hour with an inverted microscope. Early determination of antifungal sensitivity to drugs with the Flat Plate method will be useful for simple and highly accurate detection, shortening the time to obtain results without incurring additional costs. Rapid tests associated with reference antifungal susceptibility tests may be helpful in clinical laboratories to detect antifungal resistance and guide appropriate early antifungal therapy.

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