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Research Article / Araştırma Makalesi

The Investigation of the Susceptibility of Candida Species to Fluconazole, Voriconazole, and Amphotericin B by Disc Diffusion and the Microdilution Method

Candida Türlerinin Flukonazol, Vorikonazol, Amfoterisin B'ye Karşı Duyarlılıklarının Disk Difüzyon ve Mikrodilüsyon Yöntemiyle Araştırılması

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Abstract: In this study, we aimed to investigate the antifungal susceptibility profile of Candida isolates obtained in our hospital with two methods. A total of 200 Candida isolates were obtained from samples of patients in various departments of our hospital. Identification of each strain was performed using conventional (germ tube formation, microscopic morphology in corn meal-Tween 80 agar), and commercial kit API 20C (Biomerieux, France). MIC values of fluconazole (FLU), voriconazole (VORI), and amphotericin B (AMB) were evaluated according to Clinical Laboratory Standards Institute broth microdilution and disk diffusion (DD) method. Totally isolated 200 Candida species were identified as 100 C. albicans, 25 C. glabrata, 7 C. krusei, 43 C. parapsilosis, and 25 C. tropicalis. All isolates were susceptible to VORI. High resistance rate (22,5%) was detected for AMB antifungal by reference microdilution method. Between two methods, the best categorical ratio was calculated as VORI (99%), FLU (84.9%), and AMB (72,5%) respectively. Voriconazole exhibited better activity in vitro than fluconazole, even in isolates fluconazole resistant and disk diffusion method can be considered as alternative antifungal sensitivity method for VORI.
Keywords: broth microdilution, candida spp. antifungal susceptibility, antifungal, disk diffusion

Özet: Bu çalışmada hastanemizde izole edilen Candida türlerinin iki farklı yöntemle antifungal duyarlılıklarının incelenmesi amaçlanmıştır. Hastanemizde çeşitli departmanlardan gelen klinik örneklerden toplam 200 Candida türü izole edildi. Bu türler geleneksel yöntem (germ tüp oluşturma, corn-meal tween 80 besiyerinde mikroskobik görünüm) ve ticari kit API 20C (Biomerieux, France) ile tanımlandı. Bu türlerin flukonazol (FLU), vorikonazol (VORI), amfoterisin B (AMB) antifungal minimum inhibisyon konsantrasyonu (MIK) değerleri Clinical and Laboratory Standards Institute sıvı mikrodilüsyon ve disk-difüzyon (DD) yöntemine göre değerlendirildi. İzole edilen 200 Candida türü; 100 C. albicans, 25 C. glabrata, 7 C. krusei, 43 C. parapsilosis ve 25 C. tropicalis olarak tanımlandı. Bütün Candida izolatları VORI 'ye karşı duyarlı bulundu. Yüksek direnç (%22,5) oranı referans mikrodilüsyon yöntemi ile AMB antifungaline karşı tespit edilidi. İki yöntem arasında en iyi kategorik oran sırası ile VORI (%99), FLU (%84,9) ve AMB (%72,5) olarak hesaplandı. Vorikonazol, flukonazole dirençli izolatlarda bile in vitro olarak flukonazolden daha iyi aktivite göstermiştir. Ek olarak disk difüzyon yöntemi VORI için alternatif antifungal duyarlılık yöntemi olarak değerlendirilebilir.
Anahtar Kelimeler: sıvı mikrodilüsyon, candida spp, antifungal duyarlılık, antifungal, disk difüzyon

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1. Introduction

Candida infections are emerging and life-threatening infections in critically ill patients. The incidence of *Candida* infections is affected by immunotherapy, parenteral nutrition or use of broad-spectrum antibiotics, cancer, systemic infections, and indwelling vascular and urinary catheters all [1]. Azoles, polyenes, and echinocandins are the antifungals often used to treat the deep-seated and superficial fungal infections. Fluconazole (FLU) is a first-line antifungal agent due to its good tolerance and limited side effects [2]. In recent years, non-*albicans Candida* species have increased, and FLU exhibits a low susceptibility to these species [3, 4]. Voriconazole (VORI) is a derivative of fluconazole, and it is a useful alternative agent associated with a good safety profile when we detect antifungal-resistant strains in the treatment of invasive fungal infections [5]. Amphotericin B (AMB) is a macrolide polyene antifungal agent that has been used to treat fungal infections, including most strains of *Candida* spp., *Aspergillus* spp., and most other filamentous fungi, such as *Mucorales* [6]. For *in vitro* antifungal susceptibility testing of yeasts, the broth microdilution (BMD) method has been considered as a standardized method. However, this method may be inconvenient to practice in a busy routine laboratory [7]. A reliable and faster method is needed in clinics to diagnose and to treat invasive fungal disease. Disk diffusion (DD) method in agar was applied as antifungal susceptibility testing method as suggested in M44-A2 document of the Clinical Laboratory Standards Institute (CLSI) [8]. Because this test is practical and easy, it may be used in public and private clinical laboratories routinely.

The aim of this study was to compare the result of amphotericin B, fluconazole, voriconazole, susceptibility testing by DD and the CLSI reference method for clinical *Candida* isolates.

2. Materials and Method

Candida isolates

A total of 200 *Candida* isolates were obtained from blood (n=90), urine (n=82), respiratory tract (n=17), and soft-tissue (n=11) samples of patients in various departments of our hospital. Identification of each strain was performed using conventional (germ tube formation, microscopic morphology in corn meal-Tween 80 agar) and commercial kit API 20C (Biomerieux, France), and automatic identification system MALDI-TOF MS (Matriks assisted laser desorption ionization time of flight mass spectrometry) (VITEK-MS, Biomerieux, Marcy I'Etoile, France). The study was approved by the ethics board of the medical faculty.

Antifungal Agents and Susceptibility Testing

Broth Microdilution Method

We performed the BMD according to the CLSI M27-A3 reference method [7]. RPMI 1640 medium (Sigma Chemical Co, St Louis, MO, USA) with L-glutamine, without bicarbonate; was used in the microdilution method. Stock solutions of FLU (Pfizer Pharmaceuticals Group, New York, NY, USA) were prepared in distilled water, and the final concentrations of FLU were determined to be 0.125–64.00 μg/ml. VORI (Pfizer, Barcelona, Spain) and AMB (Sigma Chemical, Milan, Italy) were dissolved in in dimethyl sulfoxide (DMSO), and the final concentrations were prepared to be 0.03–32.00 μg/ml for AMB and 0.03–16.00 μg/ml for VORI.

FLU and VORI minimum inhibition concentration (MIC) breakpoints were evaluated according to CLSI M27-S4 reference method [9]. For AMB, the MIC breakpoints are follows: ≤1 as Susceptible (S), >1 resistant [10]. Quality control was performed in both tests in accordance with the CLSI document M27-A3 using *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019.

Disk-diffusion method; Antifungal susceptibility test was performed by following Clinical and Laboratory Standards Institute CLSIM44-A2 disk diffusion method. [8].The standard medium used for disk diffusion test was Mueller-Hinton agar supplemented with 2% dextrose and 0.5 µg/ml methylene blue. The agar surface was inoculated by using a swab dipped in a cell suspension adjusted to the turbidity of a 0.5 McFarland standard. Antifungal disks of FLU (25 µg/disk), VORI (1 µg/disk), and AMB (10 µg/disk) (procured from Hi-media, Mumbai) were placed on to the surface of inoculated agar plates, and the zone was measured and evaluated after 24 hours of incubation. If insufficient growth was observed after 24 hours of incubation, the incubation period was extended to 48 hours. The interpretive criteria for the FLU and VORI DD tests were those of the CLSI: susceptible (S), zone diameters of ≥19 mm (FLU) and ≥17 mm (VORI); susceptible-dose dependent (SDD), zone diameters of 15–18 mm (FLU) and 14–16 mm (VORI); and resistant (R), zone diameters of ≤14 mm (FLU) and ≤13 mm (VORI) [8]. DD breakpoints for AMB, S ≥15 mm; (SDD) 10–14 mm; and ≤9 mm R-zone diameters [11].

In this present study, we preferred the following definitions of error: When susceptibility testing was defined resistance by DD and but susceptible according to BMD, the result was explained as major errors (MEs). Very major errors (VMEs) were classified as results of susceptible by DD and resistance by BMD. Minor errors were defined when a result of susceptible or resistance existed in one of the tests and a result of SDD existed in the other method [12].

3. Results

A total of 200 *Candida* isolates were defined as 100 *C. albicans*, 25 *C. glabrata*, 7 *C. krusei*, 43 *C. parapsilosis*, and 25 *C. tropicalis*. Antifungal MIC values using BMD are shown using BMD in Table1. For FLU, VORI, and AMB, geometric mean (GM) MICs were detected as 0.28, 0.07, and 0.77 µg/ml, respectively. All of the *Candida* isolates (*C. glabrata isolates* out of scope) except one *C. parapsilosis* R and one *C. albicans* with one *C. parapsilosis* SDD were determined to be susceptible to VORI (86%) by using BMD. For VORI categorical agreement between BMD and disk diffusion at 24 h was excellent (99%), with 1 VMEs, no MEs and 1 minor errors. For FLU, while one *C. glabrata* and four *C. tropicalis* and three *C. parapsilosis* isolates were detected to be resistant by using BMD. For FLU categorical agreement between BMD and disk diffusion at 24 h was very good (84.5%), with 3 VMEs, 2 MEs and 25 minor errors. For AMB, 45 (22,5%) of 200 resistance was detected using BMD according to acceptable MIC values and the categorical agreement between the DD and the BMD results at 24 hours was 72,5% with 32 VMEs, 1 MEs, and 24 minor errors.

Table 1. In vitro susceptibilities of Candida spp. to fluconazole,voriconazole and amphotericin B as determined by CLSI broth microdilution method.

		BMD			
		MIC RANGE	**MIC50**	**MIC90**	**GM**
***C. albicans* (n=100)**	FLU	0,06-1	0,125	0,25	0,12
	VOR	0,06-0,25	0,06	0,06	0,06
	AMB	0,125-4	0,5	1	0,54
***C.glabrata* (n=25)**					
	FLU	0,125-64	2	8	1,94
	VOR	0,05-16	0,06	0,5	0,11
	AMB	0,25-4	1	2	1,12
***C.krusei* (n=7)**					

	FLU	2-16	8	16	1
	VOR	0,06-0,125	0,06	0,06	0,07
	AMB	0,5-2	1	1	1
***C.parapsilosis* (n=43)**					
	FLU	0,06-8	0,5	2	0,67
	VOR	0,06-0,25	0,06	0,125	0,08
	AMB	0,25-32	1	4	1,07
***C.tropicalis* (n=25)**					
	FLU	0,06-64	0,25	1	0,33
	VOR	0,06-16	0,06	0,125	0,08
	AMB	0,125-32	1	2	1,21

(FLU); Fluconazole, (VOR); Voriconazole, (AMB); Amphotericin B MIC : Minimal inhibitory concentration

DD method; *C.albicans* isolates were found as susceptible against all three antifungals. For VORI, FLU and AMB; GM were detected as 41%, 33%, 18%, respectively and VORI showed best antifungal susceptibility against all *Candida* species using DD. Comparisons of the results obtained by two methods are shown in Table2. by interpretive category.

Table 2. Comparison of interpretive categories and rates of interpretive agreementü

		Isolates				% Discrepancy [a]			
Antifungal	**Method**	**S**	**S-DD**	**R**		**Minor**	**Major**	**Very Major**	**% Categorical Agreement**
Fluconazole	DD	184	6	10		9(4.5%)	8 (4%)	1 (0.5%)	182 (91%)
	BMD	194	4	2					
Voriconazole	DD	200	-	-		-	-	-	200 (100%)
	BMD	200	-	-					
AmphotericinB	DD	176	22	2		6 (3%)	24(12%)	-	170 (85%)
	BMD	159	-	41					

***S,** susceptible; **I**, intermediate; **R**, resistant**; S-DD,** susceptible-dose dependent**; DD**;Disk Diffusion **BMD**; Broth microdilution*
***a** Minor discrepancies, susceptible-dose dependant by one method but susceptible or resistant by the other; major discrepancies, resistant by the test method but susceptible bythe reference test; very major discrepancies, susceptible by the test method but resistant by the reference test.*

4. Discussion

In this study, we determined the antifungal susceptibility profile of *Candida* isolates obtained from fungal infections in our hospital by using BMD and disk diffusion methods. The best categorical agreement between the DD and the BMD was determined from VORI (99%), FLU (84,9%), and AMB (72,5%).

Many studies have been performed for detecting FLU *in vitro* activity against *Candida* species, and the lowest MICs were observed for *C. albicans*; the highest MICs were observed for non-*albicans* isolates using CLSI BMD [13-15]. Our finding supported this result, we observed that all *C. albicans* isolates had lowest MIC values than non-*albicans* isolates against FLU with BMD method. Disk diffusion method showed 84,5% agreement with the reference method for fluconazole against *Candida* isolates. This discrepancy can be attributed to the difficulty

of determining antifungal endpoint by DD method. This method is based on visual reading of minimum inhibitory concentration values . The partial growth inhibition makes it difficult to determine FLU MIC values accurately [16].

VORI showed lower MIC values than FLU MIC values against most species of *Candida* with few exceptions [17]. In this present study, when we compared FLU susceptibility, VORI showed an excellent *in vitro* activity (86%) for all *Candida* isolates (*C. glabrata* isolates out of scope). In our study, we observed FLU resistant, VORI-susceptible isolates. For instance, one *C. glabrata*, four *C. tropicalis* and *three C. parapsilosis* isolates were detected to be resistant against FLU, but all *Candida* species (except one *C. parapsilosis* R and one *C. albicans* with one *C. parapsilosis* SDD) were found to be susceptible against VORI. FLU-resistant, and VORI-susceptible isolates may be attributed to enhanced binding of this triazole to the target enzyme compared to fluconazole [18,19]. Additionally, strong categorical agreement was detected between the DD and the BMD for two antifungals, with this results; DD testing appears to be a promising method for detecting VORI *in vitro* activity against *Candida* spp. These findings are consistent with those of other investigations [20, 21].

Methodology problems can be detected for determining AMB DD zone and BMD MIC values because of the narrow gap between the susceptible and resistant *Candida* isolates [22]. In our study, for AMB 45 (22,5%) isolates were detected to be resistance using BMD method and the categorical agreement between the DD and the BMD results were calculated to be 72,5% with 32 VMEs, 1 MEs, and 24 minor errors. Four isolates except all of the *C. albicans* isolates were found to be susceptible to AMB, and the agreement rate for the two methods was found to be 96% for AMB.

5. Conclusion

Conclusively, the percentage agreement between the BMD and DD test for VORI against *Candida* species was excellent and can be used for detecting VORI antifungal susceptibility against *Candida* species routinely in a laboratory. In addition; VORI showed very potent activity against fluconazole-resistant isolates. Due to the narrow gap between susceptible and resistant *Candida* isolates, we encountered a methodology problem when we evaluated AMB activity *in vitro*.

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