Activities of amphotericin B, fluconazole and voriconazole against *Candida* bloodstream isolates determined by broth microdilution and disk diffusion methods

Amfoterisin B, flukonazol ve vorikonazollünün kan kültürlerinden izole edilen *Candida* türlerine karşı antifungal aktivitesinin mikrodilüsyon ve disk diffüzyon yöntemleri ile belirlenmesi

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ÖZET

Amaç: *Candida* türlerinin neden olduğu invaziv fungal enfeksiyonlar önemli ölçüde artmıştır. Kandidemiler sadece mortalite ile ilişkili olmayıp, aynı zamanda hastanede kalış süresinin uzamasına ve tıbbi bakım maliyetinin artmasına da sebep olmaktadır. *Candida albicans* ve non-albicans *Candida* türlerinin neden olduğu kandidemilerde, hastaların klinik bulgularının aynı olmasına rağmen bu türlerin antifungal ilaçlara duyarlılıkları da farklıdır. Bu çalışmada; *Candida* kan izolatlarının antifungal duyarlılık verilerini sunmaktadır.

Yöntem: 35 *Candida albicans*, 25 *Candida* tropicalis, 15 *Candida parapsilosis*, 8 *Candida glabrata*, 4 *Candida krusei* ve 3 *Candida kefyr* olmak üzere toplam 90 *Candida* izolatı incelendi. Bu türlerin amfoterisin B, flukonazol ve vorikonazole karşı antifungal duyarlılıkları %2 glukozla zenginleştirilmiş RPMI 1640 agar besiyeri kullanılarak mikrodilüsyon yöntemi (CLSI M27-A3) ve 0,5 µg/mL metilen mavisi ve %2 glukozla zenginleştirilmiş Mueller-Hinton agar besiyeri kullanılarak disk diffüzyon (CLSI M44-A2) yöntemi ile belirlendi.

Bulgular: *Candida* izolatları mikrodilüsyon yöntemi ile %87,7 oranında, disk diffüzyon yöntemi ile %82,2 oranında flukonazole duyarlı bulundu.

ABSTRACT

Objective: Invasive fungal infections caused by *Candida* species have increased significantly. Candidemia is not only associated with a mortality, but also extends the duration of hospital stay and increases the cost for medical care. Although, the clinical presentations of the patients with candidemia caused by *Candida albicans* and non-albicans *Candida* species are indistinguishable, the susceptibilities to antifungal agents of these species are different. This study presents data on antifungal susceptibility profiles of *Candida* bloodstream isolates.

Method: We tested a total of 90 strains, including 35 strains of *Candida albicans*, 25 strains of *Candida tropicalis*, 15 strains of *Candida parapsilosis*, 8 strains of *Candida glabrata*, 4 strains of *Candida krusei* and 3 strains of *Candida kefyr*. Susceptibility to amphotericin B, fluconazole and voriconazole was determined by Clinical Laboratory Standards Institute broth microdilution method (CLSI M27-A3) using RPMI 1640 as test medium supplemented with 2% glucose and disk diffusion methods were performed according to CLSI M44-A2 using methylene blue (0.5 µg/mL) and glucose (2%) enriched Mueller-Hinton agar.

Results: In this study, 87.7%, 82.2% of *Candida* isolates were susceptible to fluconazole with the broth

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Mikrodilüsyon yöntemi ile bu izolatların %6,6'sı (%8,5 *C. albicans*, %4,0 *C. tropicalis* ve % 25,0 *C. glabrata*) flukonazole karşı doza bağlı duyarlı, %5,5 ise dirençli olarak belirlendi. Dirençli suşların dördü *C. krusei* (%100), biri ise *C. albicans* (%2,8) idi. Disk diffüzyon yöntemi ile *Candida* suşlarının %12,2'si doza bağlı duyarlı (%17,1 *C. albicans*, %12,0 *C. tropicalis* and %25,0 *C. glabrata*) olarak, %5,5'i [bir izolat *C. albicans* (%2,8), dört izolat *C. krusei* (%100)] ise flukonazole dirençli olarak belirlendi. Bütün suşlar amfoterisin B ve vorikonazole duyarlı olarak bulundu.

Sonuç: Amfoterisin B ve vorikonazollün *Candida* izolatlarına karşı in-vitro aktivitesinin yüksek olduğu belirlendi. Antifungal ilaçlara direnç artmaktadır ki bundan dolayı fungal patojenlerin duyarlılık profillerini değerlendirmek giderek önem kazanmaktadır.

Anahtar Kelimeler: Antifungal duyarlılık, flukonazol, kan izolatları

microdilution method and the disk diffusion method respectively, based on CLSI breakpoints. A further 6.6% were classified as susceptible-dose-dependent (8.5% *C. albicans*, 4.0% *C. tropicalis* and 25.0% *C. glabrata*) and fluconazole resistance was detected in 5.5% of all isolates by microdilution method. Four isolates of these strains were *C. krusei* (100%) and one strain was *C. albicans* (2.8%). 12.2% of *Candida* spp. were classified as susceptible-dose-dependent (17.1% *C. albicans*, 12.0% *C. tropicalis*, and 25.0% *C. glabrata*) and fluconazole resistance was detected in 5.5% of all isolates. Four isolates of these strains were *C. krusei* (100%) and one strain was *C. albicans* (2.8%) by disk diffusion method. All isolates were susceptible to amphotericin B and voriconazole using by two methods.

Conclusion: Voriconazole and amphotericin B were active in-vitro against yeasts. As antifungal drug resistance may become more frequent, it is increasingly important to evaluate current antifungal susceptibility profiles of fungal pathogens.

Key Words: Antifungal susceptibility, fluconazole, bloodstream isolates

INTRODUCTION

A progressive increase in the frequency of candidemia has been observed, particularly among patients receiving antibiotics, immunosuppressive therapy, or parenteral nutrition, as well as among patients exposed to invasive medical procedures such as intravascular catheter, hemodialysis and abdominal surgery (1-3).

Candidemia have been associated with significant mortality, especially among critically ill patients. The attributable mortality of candidemia has been estimated at 25-38%. In addition, an increase of 30 days in the length of hospital stay among patients surviving these infections has been demonstrated. The economic impact of these infections is also important (3-6).

Although *Candida albicans* is still considered the most frequently isolated species of candidemic patients, the emergence of non-albicans *Candida* species is clearly a concern. The resistance of non-albicans *Candida* isolates to currently available antifungal drugs represents a major challenge for future empirical therapeutic and prophylactic strategies. Therefore, species directed therapy should be administered for fungemia according to the species identified and its antifungal susceptibility pattern (1, 3).

Candida species have various degrees of susceptibility to frequently used antifungal drugs. For instance, *Candida lusitaniae* is relatively resistant to amphotericin B, *C. krusei* is intrinsically resistant to fluconazole, *C. glabrata* is less susceptible or has a higher MICs to it than other *Candida* species. This phenomenon illustrates the importance of identification and surveillance of *Candida* species in the clinical settings (7).

Large-scale surveillance for bacteremia and fungemia has been conducted world-wide by various organizations, including the Centers for Disease

Control and Prevention (CDC) and the National Epidemiology of Mycoses Survey (NEMIS). These surveys provide evidence that the prevalence of azole-resistant fungi is increasing (8).

The antifungal susceptibility testing of pathogenic fungi can manage the selection of adequate therapy and also provide an estimate of antifungal efficacy. Monitoring of drug resistance development can predict therapeutic outcome and therapeutic potential of untested compounds (9).

With the increasing incidence of yeast infections and the emergence of resistant strains to antifungal drugs, it has become essential for diagnostic laboratories not only to isolate and identify *Candida* species but also to perform routine susceptibility testing (10).

The Clinical and Laboratory Standards Institute (CLSI) provides recommendations for broth microdilution antifungal susceptibility testing of yeasts, a method that is reproducible and presents good clinical correlation. However, this method is labor-intensive and unsuitable for most routine diagnostic laboratories (10).

Correlation between results generated by the CLSI microdilution standard reference method and a disk diffusion assay using Mueller-Hinton agar supplemented with 2% glucose and 0.5 μg/mL methylene blue agar has been documented by several investigators. Breakpoints for susceptibility of fluconazole and voriconazole are now available for disk diffusion assay which have the advantages of being easy to perform, accurate, inexpensive and suitable for routine laboratories (10).

The purpose of this study was to test *Candida* species obtained from blood culture against amphotericin B, fluconazole and voriconazole by using the broth microdilution and disk diffusion methods.

MATERIALS and METHODS

Candida Isolation and Identification

This study was designed in Mycology Laboratory, Microbiology and Clinical Microbiology Department of Medical Faculty between 2011 to 2013 years. A total of 90 yeast isolates were tested. All the organisms were clinical blood isolates obtained from patients hospitalized. The isolates were identified by standard conventional methods. For species typing of the isolates, germ tube and chlamydospore production tests were performed. The carbohydrate assimilation patterns of all isolates were studied using the API 20 C AUX system (Biomerieux) according to the manufacturer's procedure. The yeasts were maintained on Sabouraud glucose agar slants, stored at 4 °C, until used in the study. Prior to testing, each strain was subcultured on Sabouraud agar for 24 h at 35 °C to ensure viability. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were tested each time a set of clinical isolates was evaluated (11).

Antifungal Susceptibility Test

Microdilution Method: Reference antifungal susceptibility testing of *Candida* spp. was performed using the microdilution method described in CLSI M27-A3 (12). Reference powders of amphotericin B (Sigma), flukonazole (Pfizer) and voriconazole (Pfizer) were used. Stock solutions were prepared with a concentration 10 fold the final concentration and diluted with RPMI 1640 (Sigma), with L-glutamine, without bicarbonate, supplemented with 2% dextrose and buffered to pH 7.0 with 0.165 N-morpholinopropanesulfonic acid (MOPS) to obtain twice the final concentration.

Disk Diffusion Method: Disk diffusion testing of amphotericin B, flukonazole and voriconazole was performed as described by CLSI document M44-A2 (13). In our study; disks containing; 25 μg of fluconazole (Becton Dickinson, Sparks, MD), 1 μg of voriconazole (Becton Dickinson, Sparks, MD)

and 20 μg of amphotericin B (MAST Diagnostics) were used. Initially, a yeast inoculums suspension adjusted to match a 0.5 McFarland density standard was prepared. A sterile cotton swab moistened with inoculums suspension was used to apply each organism to be tested on a 90 mm diameter plate containing Mueller-Hinton agar supplemented with 2% glucose and 0.5 μg/mL methylene blue. The plates were allowed to dry for 5-15 min before the disks to be placed in the agar. The plates were incubated for 18-24 h at 35-37 °C, and the slowly growing isolates could be read after 24 h incubation. All inhibition zone diameters generated by disk diffusion tests were read and recorded (10, 14, 15). The interpretive criteria for the fluconazole disk test were dz ≥ 19 mm: S, 15< dz <18 mm: S-DD, dz≤ 14 mm: R, for voriconazole disk test were dz ≥ 17 mm: S, 14< dz <16 mm: S-DD, dz≤ 13 mm: R, for amphotericin B disk test dz≤ 10 mm: R (13, 16).

RESULTS

A total of 90 *Candida* spp. were isolated from clinical blood samples obtained from patients hospitalized. The most species isolated was *C. albicans* followed by *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei* and *C. kefyr* (Table 1).

Table 1. Species distribution of *Candida* isolated from blood cultures

Species	Number of isolates	% of isolates
C. albicans	35	39.0%
C. tropicalis	25	28.0%
C. parapsilosis	15	16.5%
C. glabrata	8	8.8%
C. krusei	4	4.4%
C. kefyr	3	3.3%
Total	**90**	**100**

Table 2. In vitro susceptibilities of *Candida* bloodstream isolates to fluconazole, voriconazole and amphotericin B by using microdilution method

Species and Number	Antifungal agent	MIC range (μg/mL)	MIC$_{50}$ (μg/mL)	MIC$_{90}$ (μg/mL)
C. albicans -35	Fluconazole	0.125-64	0.5	16
	Voriconazole	0.125-1	0.25	0.5
	Amphotericin B	0.03-0.5	0.125	0.25
C. tropicalis -25	Fluconazole	0.125-32	0.5	2
	Voriconazole	0.125-1	0.25	0.5
	Amphotericin B	0.03-0.5	0.125	0.25
C. parapsilosis -15	Fluconazole	0.125-4	0.25	0.5
	Voriconazole	0.125-0.5	0.125	0.5
	Amphotericin B	0.03-0.5	0.06	0.25
C. glabrata -8	Fluconazole	0.125-32	0.5	1
	Voriconazole	0.125-1	0.25	0.25
	Amphotericin B	0.06-1	0.5	1
C. krusei -4	Fluconazole	64	64	64
	Voriconazole	0.25-1	0.25	0.5
	Amphotericin B	0.125-1	0.25	0.5
C. kefyr -3	Fluconazole	0.125-0.25	0.25	0.25
	Voriconazole	0.125-0.25	0.125	0.25
	Amphotericin B	0.03-0.125	0.06	0.125

Table 2 shows MIC, MIC_{50} and MIC_{90} values exhibited by the three antifungal drugs tested against *Candida* spp. strains. *Candida* isolates were all susceptible to amphotericin B and voriconazole. Six *Candida* strains were susceptibility dose dependent (DD) to fluconazole (16-32 µg/mL). Three of these strains were *C. albicans*, one *C. tropicalis* and two *C. glabrata*. Five strains (four *C. krusei* and one *C. albicans*) were resistant to fluconazole (≥ 64 µg/mL).

In-vitro antifungal activity of fluconazole, voriconazole and amphotericin B against *Candida* species by using disk diffusion method were summarized in Table 3. Five strains (four *C. krusei* and one *C. albicans*) were resistant to fluconazole (dz≤ 14 mm: R). Eleven *Candida* isolates (six *C. albicans*, three *C. tropicalis* and two *C. glabrata*) were susceptibility dose dependent (DD) to fluconazole (15< dz <18 mm: S-DD) by disk diffusion method. Five fluconazole-sensitive *Candida* strains that determined by the microdilution method was found to be dose-dependent susceptibility in the disk diffusion method.

DISCUSSION

The importance of *Candida* spp. as etiologic agents of bloodstream infections in hospitalized patients is well established. *C. albicans* isolates usually are susceptible to commonly used azoles such as itraconazole or fluconazole. Nevertheless within the past decade, the proportion of infections caused by non-albicans *Candida* species has increased exponentially. Nearly one half of the cases of hematogenous candidiasis are now reported to be the result of *Candida* species other than albicans. The reasons for the shift are unclear and probably multifactorial. Among others, routine prophylactic use of antifungal agents such as fluconazole in immunocompromised patients can be implicated in the shift of species with known reduced susceptibility such as *C. glabrata* or *C. krusei*, which is intrinsically resistant to fluconazole (4, 16). Several of these species exhibit resistance to traditional triazole antifungals like fluconazole, and may also demonstrate cross-resistance to newer triazoles. This makes it imperative to perform both

Table 3. In vitro antifungal activity of fluconazole, voriconazole and amphotericin B against *Candida* bloodstream isolates by using disk diffusion method

Candida species	Number of isolates	% of isolates	Fluconazole			Voriconazole			Amphotericin B		
			S	SDD	R	S	SDD	R	S	SDD	R
C. albicans	35	39.0	28 80.0%	6 17.1%	1 2.8%	35 100%	-	-	35 100%	-	-
C. tropicalis	25	28.0	22 88.0%	3 12.0%	-	25 100%	-	-	25 100%	-	-
C. parapsilosis	15	16.5	15 100%	-	-	15 100%	-	-	15 100%	-	-
C. glabrata	8	8.8	6 75.0%	2 25.0%	-	8 100%	-	-	8 100%	-	-
C. krusei	4	4.4	-	-	4 100%	4 100%	-	-	4 100%	-	-
C. kefyr	3	3.3	3 100%	-	-	3 100%	-	-	3 100%	-	-
Total	**90**	**100**	**74 82.2%**	**11 12.2%**	**5 5.5%**	**90 100%**	**-**	**-**	**90 100%**	**-**	**-**

speciation and antifungal susceptibility testing of all yeast fungi isolated from bloodstream or otherwise (6).

In this study, we isolated 90 strains from clinical blood samples with higher rate of *C. albicans* (39.0%), followed by *C. tropicalis* (28.0%), and *C. parapsilosis* (16.5%). The similar rates have been presented in other studies (1, 2, 6, 18-23). Surveillance studies such as NEMIS, Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE), and a SENTRY Antimicrobial Surveillance Program revealed that *C. albicans* accounted for 54%, 52%, and 53% of *Candida* isolates, respectively (8, 16, 24).

Currently, in vitro susceptibility tests can allow very important guidelines for candidiasis treatment, but the standard susceptibility test (CLSI M27-A3) is not always readily available in regular laboratories and is very time consuming, in opposition to the other more simple techniques such as E-test and disk diffusion test. The major feature of these agar based tests is that they can allow a quick answer concerning *Candida* resistance to antifungal agents, preventing unnecessary patients drug abuse (9, 10, 14, 21, 25).

Fluconazole is a useful antimycotic, and is commonly used for treatment of candidemia. Strains of *C. albicans* primarily resistant to fluconazole have been identified in clinical blood isolates by several authors, varying in frequency between 0% and 38% (26-29).

In our present study, with the broth microdilution method, 87.7% of *Candida* isolates were susceptible to fluconazole based on CLSI breakpoints, and a further 6.6% were classified as susceptible-dose-dependent (8.5% *C. albicans*, 4.1% *C. tropicalis* and 25% *C. glabrata*). Fluconazole resistance was detected in 5.5% of all isolates. Four isolates of

these strains were *C. krusei* (100%) and one strain was *C. albicans* (2.8%).

In this study, with the disk diffusion method, 82.2% of *Candida* isolates were susceptible to fluconazole based on CLSI breakpoints, and a further 12.2% were classified as susceptible-dose-dependent (17.1% *C. albicans*, 12.0% *C. tropicalis* and 25% *C. glabrata*). Fluconazole resistance was detected in 5.5% of all isolates. Four isolates of these strains were *C. krusei* (100%) and one strain was *C. albicans* (2.8%).

Clinical backgrounds revealed that *C. krusei* infection was seen almost exclusively as a complication of hematological malignancy. All patients who had *C. krusei* infections had been treated orally with fluconazole for the prevention of intestinal mycoses. Consequently, fluconazole-resistant *C. krusei* remained in the intestine, from which the organisms are likely to have become translocated into the circulation. Thus, it is seems that the presence of antimycotic resistant strains of fungi is always conceivable in patients with hematological malignancies (8).

In our study, 100% of *C. krusei* strains were classified as in vitro resistant to fluconazole. The resistance rates to fluconazole were higher than 80% usually reported by most authors in other studies. Regardless of the results obtained with in vitro studies, *C. krusei* strains should be considered as inherently resistant to fluconazole (4, 21, 23, 30, 31).

C. glabrata, a species that easily acquires azoles drug resistance, is represented in European surveillance data of the 1990s at proportions in the range 9%-16%, depending on the geographic location (30). It has been reported that continuous exposure to azoles seems to have a major impact on developing resistance to fluconazole in *Candida*

species, especially for *C. glabrata* (7). In this study, *C. glabrata* accounted 8.8% of bloodstream isolates and a further 25% were classified as susceptible-dose-dependent to fluconazole by two methods.

Blood stream infection due to *C. kefyr* is uncommon, but there have been some reports in immunocompromised patients and resistance to antifungals in the literature (9, 18, 23). In the present study, 3.3% of *Candida* isolates were *C. kefyr* of which were sensitive fluconazole, voriconazole and amphotericin B.

In our study, all *C. parapsilosis* isolates were susceptible to three antifungals and one *C. tropicalis* (4.1%) were classified as susceptible-dose-dependent to fluconazole by microdilution method. Three *C. tropicalis* isolates (12.0%) were susceptible-dose-dependent to fluconazole by disk diffusion method. The increasing rate of reduced susceptibility to fluconazole in *C. tropicalis* has considerable clinical importance, because this species is one of the most frequently isolated non albicans *Candida* species. Furthermore, *C. tropicalis* develops drugs resistance in presence of fluconazole much more rapidly than *C. albicans* (7).

Amphotericin B was the first systemic antifungal agent for the treatment of invasive fungal infections and has been the drug of choice; however, due to nephrotoxicity in up to 80% of the patients, use of amphotericin B has been limited. Amphotericin B is effective against *Candida* spp. that are resistant to other antifungal agents (9). In our study, all isolates were susceptible to amphotericin B. Voriconazole was the triazole with the high in vitro antifungal activity against all *Candida* strains by use two methods and all isolates were susceptible to voriconazole.

In conclusion, the successful treatment of candidemia depends on the early identification of the species and sensitivity patterns to antifungal agents. The high growing rate of non albicans *Candida* resistant to azoles confirms the importance of monitoring changes in the distribution of pathogenic *Candida* species. The sensitivity pattern of *Candida* species as revealed in this study shows that amphotericin B and voriconazole were active antifungal against *Candida* spp.

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