Research Article (Araştırma Makalesi)

Determination of Resistance Rates of *Candida albicans* Species Isolated from Sterile Body Fluids to Triazoles by Microdilution Method

Steril Vücut Sıvılarından İzole Edilen Candida albicans Türlerinin Mikrodilüsyon Yöntemiyle Triazollere Direnç Oranlarının Belirlenmesi

Filiz ORAK[1]

[1] Kahramanmaras Sutcu Imam University School of Medicine, Department of Microbiology, Kahramanmaras, Turkey

Özet

Amaç: Azollerin profilaksi veya tedavi amacıyla yaygın ve tekrarlayan kullanımı *Candida albicans (C.albicans)* türlerinde direnç gelişimine yol açmıştır.

Gereç ve Yöntemler: Bu çalışmada, steril vücut sıvısı kültürlerinden izole edilen *C.albicans* izolatlarının flukonazol, itrakonazol ve vorikonazole *in vitro* direnç oranlarının belirlenmesi amaçlanmıştır. Tür düzeyinde tanımlama, geleneksel tanı yöntemleri ve BD Phoenix 100 (BD, ABD) otomatik tanımlama sistemi kullanılarak yapıldı. Antifungal duyarlılık testi, %2 glukoz içeren RPMI kullanılarak mikrodilüsyon yöntemiyle değerlendirildi. Triazoller olan flukonazol, itrakonazol ve vorikonazol için bulanıklığın belirgin (≥%50) olarak azaldığı kuyucuktaki konsantrasyon, MİK$_{50}$ değeri olarak belirlendi. Çalışmada kontrol suş olarak *C.albicans* ATCC 90028 kullanıldı.

Bulgular: *C.albicans* izolatlarının %88.2'si (n=45) yoğun bakım ünitelerinden alınan örneklerden izole edildi. Örneklerin 26 (%50.9)'sı yaş ortalaması 68.6±18.29 yıl olan kadın hastalardan, 25 (%49)'i yaş ortalaması 62.22±21.08 yıl olan erkek hastalardan alındı. İzolatların 48 (%94.1)'i kan,1 (%1.96)'i beyin omurilik sıvısı, 1 (%1.96)'i steril vücut sıvısı, 1(%1.96)'i peritoneal sıvı kültüründen tanımlandı. Antifungal duyarlılık test sonuçlarına göre türlerin 7 (%12.2)'si flukonazol, 14 (%27.4)'ü itrakonazol ve 5 (%9.8)'i vorikonazole dirençli bulundu. Toplam 6 (%11.7) örnekte ise çapraz dirence rastlandı.

Sonuç: *C.albicans* izolatlarında triazollere direncin yanısıra diğer azollere karşı da çapraz direnç saptanmıştır. Bu nedenle uygun tedavi için antifungal duyarlılık test sonuçları dikkate alınmalıdır.

Anahtar kelimeler: Antifungal ilaç direnci, Candida albicans, Triazol, Vücut sıvısı

Abstract

Objective: Widespread and repeated use of azoles for prophylaxis or therapy has led to the development of resistance in *Candida albicans (C.albicans)* species. In this study, it was aimed to determine the *in vitro* resistance rates of *C.albicans* isolates isolated from sterile body fluid cultures to fluconazole, itroconazole and voriconazole.

Material and Methods: Fifty one *C.albicans* species isolated from sterile body fluid cultures of patients hospitalized in various clinics between January 2020 and April 2021 were included in the study. Species-level identification was assessed using conventional methods and the BD Phoenix 100 (BD, USA) automated identification system. Antifungal susceptibility testing was performed by microdilution method using RPMI medium containing 2% glucose. For the triazoles fluconazole, itroconazole and voriconazole, the concentration in the well at which turbidity was reduced significantly (≥50%) was determined as the MIC50 value. *C.albicans* ATCC 90028 was used as control strain.

Results: Eighty-eight point two percent (n=45) of *C.albicans* isolates were isolated from samples taken from intensive care units. Twenty-six (50.9%) of the samples were taken from female patients with a mean age of 68.6±18.29 years, and 25 (49%) from male patients with a mean age of 62.22±21.08 years. Of the isolates, 48 (94.1%) was identified from blood, 1 (1.96%) cerebrospinal fluid, 1(1.96%) sterile body fluid, 1(1.96%) peritoneal fluid culture. According to the antifungal susceptibility test results; 7 (12.2%) of the species were resistant to fluconazole, 14 (27.4%) to itroconazole, and 2 (3.9%) to 5 (9.8%) voriconazole. Cross-resistance was detected in a total of 6 (11.7%) samples.

Conclusion: In addition to resistance to triazoles, cross-resistance was also detected against other azoles in *C.albicans* isolates. Therefore, antifungal susceptibility test results should be taken into account for appropriate treatment.

Keywords: Antifungal drug resistance, Body fluid, *Candida albicans*, Triazole

Yazışma Adresi: Filiz ORAK, Sütçü İmam Üniversitesi Tıp Fakültesi Mikrobiyoloji Anabilim Dalı, Kahramanmaraş, Türkiye
Telefon: +905063371046
Email: drfilizorak@hotmail.com
ORCID No (Sırasıyla): 0000-0001-5153-7391
Geliş tarihi: 13.07.2021
Kabul tarihi: 31.08.2021
DOI: 10.17517/ksutfd.971050

INTRODUCTION

Clinical symptoms of systematic candida infections are generally nonspecific and it has been reported that due to the delay in starting antifungal treatment mortality rates increase (1). In most clinics, *Candida albicans* (*C. albicans*) is the main reason for candidiasis. As *C. albicans*, found in oral, conjunctival, gastrointestinal, and genitourinary microbiota, can cause superficial infection, it can also cause septicemia as a result of invasion.

According to the Centers for Disease Control and Prevention data, *Candida* species are the third most isolated microorganisms from blood in the USA (2). In Turkey, among the factors causing bloodstream infection *Candida* spp. is ranked as third and the *Candida albicans* is ranked as fourth (3).

Preventing and treating the infections caused by the *Candida* species usually necessitates long-term drug use (4). Azoles, polyenes, and echinocandins are used as the first-line therapy for invasive candidiasis in many hospitals. But in severe infections, due to infusion-related side effects and dose-limiting nephrotoxicity, the use of polyenes is limited. Azoles inhibit fungus-specific lanosterol 14-α-demethylase and prevent the synthesis of fungus-specific ergosterol. This provides an advantage in terms of use. Azoles are divided into two groups as imidazoles (e.g. mycanazole and ketoconazole) and triazoles (e.g. itraconazole, fluconazole, voriconazole). Fluconazole resembles ketoconazole in terms of antifungal spectrum and mechanism of action and provides a safer profile of usage. (1).

In *C. albicans* species high-level azole resistance might occur depending on the infection type and prior use of fluconazole (5). *C. albicans* can easily avoid host immune defense through its properties like forming biofilm and changing its form from yeast to hypal or pseudohyphal form. Up to 1000 times more azole resistance has been reported in the biotypes of *C. albicans* which produces biofilm (1). In addition, selection of spontaneous mutations, which reduces the susceptibility to antifungals, chromosomal abnormalities (aneuploidy), alteration in the synthesis of ergosterol and lanosterol demethylase (*Erg11p*) which is the aim of the drug, and upregulation or overexpression of efflux pumps are held responsible for azole resistance.

Overexpression of efflux pumps has been associated with azole resistance, particularly in the early stages of biofilm formation in *C. albicans* (7-9).

In this study, it was aimed to evaluate the susceptibility of *C.albicans* isolates isolated from sterile body fluids in a tertiary hospital to triazole antifungals by microdilution method.

MATERIALS AND METHODS

Identification of *Candida* isolates

In this study, *C. albicans* isolates obtained from sterile body fluid culture samples sent to Kahramanmaraş Sütçü Imam University Microbiology Laboratory for routine diagnosis between January 2020 and April 2021 were included. Blood samples were incubated in the Bact-ALERT (Biomerieux, USA) automated system. Blood culture bottles with growth signals and other sterile body fluid samples were inoculated on 5% sheep blood agar (BD, USA), EMB agar (BD, USA), and Saboraud's dextrose agar (HIMEDIA, India) and incubated at 37 ˚C. Yeast appearance was examined in microscopy with Gram staining. Germ tube test, growth on Cornmeal agar (HIMEDIA, India), chlamydospore formation, presence of pseudohyphae, and typical green colony appearance on HiCrome™ Candida Differential Agar (HIMEDIA, India) were evaluated for identification of isolates. In addition, species-level identification was performed with the BD Phoenix 100 (BD, USA) automated identification system.

Antifungal Susceptibility Test

Microdilution method was used according to the European Committee for Antibiotic Susceptibility Testing (EUCAST) recommendations for antifungal susceptibility testing. Serial dilutions of fluconazole (0.25-128 µg/mL), itroconazole (0.0156-8 µg/mL) and voriconazole (0.0156-8 µg/mL) were performed in RPMI medium containing 2% glucose on flat-bottomed microplates. *Candida* suspension was inoculated into the wells 1-11 at 0.5 McFarland turbidity setting and a final volume of 1-5x10^5cfu/mL. Distilled water was added to well 12 as a negative control.

For fluconazole (FLU), itraconazole (ITZ), and voriconazole (VOR), the concentration in the well where turbidity was significantly reduced (≥50%) was considered the M$İK_{50}$ value.

Based on clinical breakpoints, ≥4 µg/mL was evaluated as resistant (R) for FLU, ≥0.064 µg/mL R for ITZ, and ≥0.25 µg/mL R for VOR. *C.albicans* ATCC 90028 was used as the control strain in the study.

Ethics Committee Approval

This study has been approved by Kahramanmaras Sutcu Imam University Clinical Research Ethics Committee in session dated 25.05.2021 with the decision number of 03-2021/18 and informed consent from patients have been obtained. Throughout the research, the Helsinki Declaration was adhered to.

RESULTS

C.albicans strains were isolated from intensive care units (88.2%), internal clinics (3.9%), and oncology and hematology clinics (3.9%), respectively (**Figure 1**). Of the samples, 1 (1.9%) was cerebrospinal fluid, 1 (1.9%) was sterile body fluid, 1 (1.9%) was bronchoalveolar lavage, and 48 (94.1%) were blood cultures. Twenty-six (50.9%) of the samples were taken from female patients with a mean age of 68.6±18.29 years, and 25 (49%) from male patients with a mean age of 62.22±21.08 years.

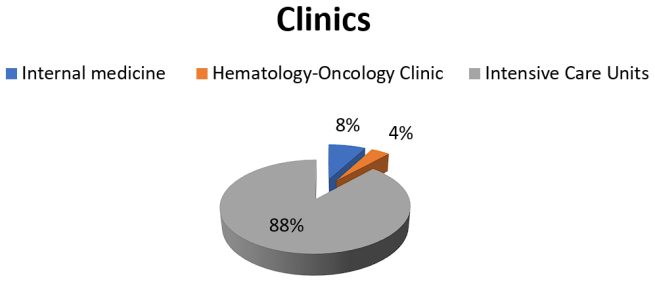

Figure 1. Distribution of C.albicans species isolated from clinics

MIC_{50} values were determined as 0.5 µg/mL for FLU, 0.0625 µg/mL for ITZ and 0.0312 µg/mL for VOR (**Table 1**).

Seven (12.2%) of *C. albicans* isolates were found to be resistant to FLU, 14 (27.4%) to ITZ, and 5 (9.8%) to VOR. Cross-resistance was found in a total of 6 (11.7%) samples (**Figure 2**).

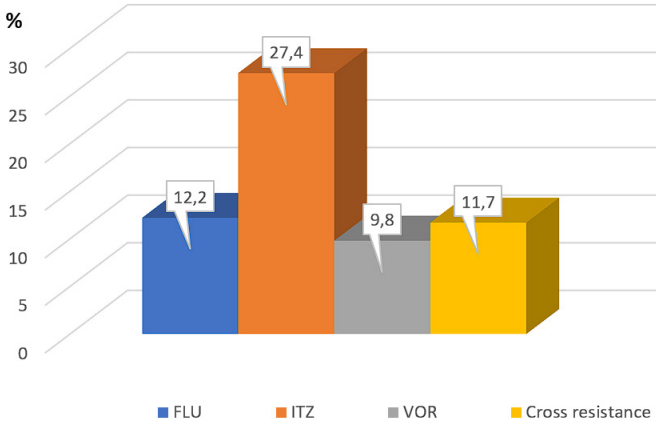

Figure 2. Resistance rates of C.albicans isolates to triazole antifungals (%)
FLU: fluconazole, ITZ: itroconazole, VOR: voriconazole

Of these; two isolates from the same patient were resistant to FLU+ITZ, one isolate to ITZ+VOR, and three isolates to FLU+ITZ+VOR. MIC_{50} values of *C.albicans* ATCC 90028 strain were evaluated as 0.25 µg/mL, 0.0156 µg/mL and 0.0156 µg/mL for FLU, ITZ and VOR, respectively.

DISCUSSION

The increased use of antifungal drugs has placed highly selective pressure on fungal species, and resistance has occurred in three ways. These are intrinsic resistance, acquired resistance, and clinical resistance (10). Acquired resistance usually develops after exposure to an antifungal agent and is reversible due to temporary

Table 1. MIC ranges, MIC50 and MIC90 values for fluconazole, itroconazole and voriconazole of *Candida albicans* isolates

Antifungal Agent	Breackpoints (µg/mL) S	R	MIC Range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	GM (µg/mL)
Fluconazole	≤2	>4	0.25-32	0.5	4	0.844
Itroconazole	≤0.064		0.0156-1	0.0625	0.25	0.053
Voriconazole	≤0.064	>0.25	0.0156-0.5	0.0312	0.25	0.033

S: Sensitive; R: Resistant; MIC: Minimum inhibitory concentration; MIC_{50}: The minimum inhibitory concentration that inhibits growth of microorganisms by 50%; MIC_{90}: Minimum inhibitory concentration that inhibits growth of microorganisms by 90%, GM: Geometric mean

adaptation in the fungus or may become permanent due to one or more chromosomal mutation (10). Clinical resistance is defined as the progression of the infection despite the initiation of treatment with a sensitive antifungal *in vitro* (11). Clinical conditions differ significantly from *in vitro* conditions (12). In particular, patients hospitalized in the intensive care unit are under the pressure of both antifungal agents and other drugs. Drug interactions could contribute to clinical resistance.

Fluconazole and drugs such as ibuprofen, pyrazinamide, amphotericin B or the flavonoid kaempferol may have a synergistic effect, while the simultaneous use of fluoroquinolones or rifampicin may have an antagonistic effect (1). For these reasons antifungal susceptibility testing plays an increasing role in cases of clinical and/or *in vitro* antifungal resistance or increased tolerance (12).

EUCAST and the Clinical Laboratory Standards Institute (CLSI) accept microdilution methods as the gold standard for antifungal susceptibility testing (13). According to EUCAST and CLSI, interpretation differences in MIC values could alter resistance results (5). In our study, antifungal susceptibility tests were evaluated according to EUCAST criteria. In a multicenter study conducted in Switzerland, *C. albicans* species isolated from blood samples at a rate of 61.9% compared to other fungal agents were evaluated according to EUCAST and CLSI criteria: FLU resistance was determined as 0.4% vs 1.6%, and VOR resistance as 0.6% vs 0.4% (14). Similarly, Lindberg et al. detected *C.albicans* as the most common (65%) fungal agent in blood cultures and found that only one of them was resistant to FLU and one isolate to VOR (15). They determined the MIC ranges as 0.12-4 µg/mL for FLU, 0.015-0.12 µg/mL for ITZ, and 0.008-0.25 µg/mL for VOR. In our study, the MIC ranges were 0.25-32 µg/mL for FLU, 0.0156-1 µg/mL for ITZ, and 0.0156-0.5 µg/mL for VOR. Dalyan et al. found that MIC values determined according to EUCAST criteria were mostly higher than CLSI criteria (16). According to Gulat et al. ITZ resistance was found in 4 of 5 fluconazole-resistant *Candida albicans* isolates and none of them reported VOR resistance according to CLSI criteria (17). In our study, the highest resistance was found to ITZ (27.4%), the lowest to VOR (9.8%), and cross-resistance was 11.7% (n=6). On the other hand, Coskun et al. found the FLU MIC ranges for *C.albicans* species isolated from blood and urine samples as 0.5-64 µg/mL and 0.25-16 µg/mL, respectively (18). In a tertiary hospital in Bulgaria, 7 of 61 *C.albicans* species isolated from blood cultures were resistant to all azoles (5).

Higher resistance in FLU and ITZ compared to VOR, in general, may be attributed to the more frequent use of these antifungal agents. FLU is a drug that does not require routine monitoring, except for invasive candidiasis, treatment of isolates with reduced susceptibility, and newborns with central nervous system diseases (19).

Although the main purpose of antifungal susceptibility testing is to determine the appropriate therapeutic agent, these methods also allow the detection of resistant isolates and the acquisition of local epidemiological data. In particular, patients hospitalized in intensive care units and receiving long-term azole therapy should be monitored due to drug side effects and the risk of selecting strains prone to drug resistance.

Conflict of Interest and Financial Status: Our study has not been financed by an institution and institution. In this study, there is no conflict of interest among the authors on any subject.

Ethics and Patient Consent: This study has been approved by Kahramanmaras Sutcu Imam University Clinical Research Ethics Committee in session dated 25.05.2021 with the decision number of 03-2021/18 and informed consent from patients have been obtained.

REFERENCES

1. Costa-de-Oliveira S, Rodrigues AG. Candida albicans antifungal resistance and tolerance in bloodstream infections: The triad yeast-host-antifungal. Microorganisms 2020;154(8):1-19.
2. Cheng MF, Yang YL, Yao TJ, Lin CY, Liu JS, Tang RB et al. Risk factors for fatal candidemia caused by Candida albicans and non-albicans Candida species. BMC Infectious Diseases 2005;5:22.
3. Erdem I, Ozgultekin A, Inan AS, Engin DO, Akcay SS, Turan G et al. Bloodstream infections in a medical-surgical intensive care unit: Incidence, aetiology, antimicrobial resistance patterns of gram-positive and gram-negative bacteria. Clin Microbiol Infect 2009;15(10):943-946.
4. Pappas PG, Kauffman CA, Andes D, Clancy CJ, Marr KA, Ostrosky-Zeichner L et al. Clinical practice guidelines for the management of candidiasis:2016 update by the Infectious Diseases Society of America. Clin Infect Dis 2015;62(4):1-50.
5. Hitkova HY, Georgieva DS, Hristova PM and Sredkova MP. Antifungal susceptibility of Candida albicans isolates at a tertiary care hospital in Bulgaria. Jundishapur J Microbiol 2019. In Press (In Press):e92079.
6. Pristov KE, Ghannoum MA. Resistance of Candida to azoles and echinocandins worldwide. Clinical Microbiology and Infection 2019;25:792-798.
7. Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. Mechanism of fluconazole resistance in Candida albicans biofilms: Phase-specific role of efflux pumps and membrane sterols. Infect Immun 2003;71:4333–4340.

8. Ramage G, Rajendran R, Sherry L, WilliamsC. Fungal biofilm resistance. Int J Microbiol 2012:528521.
9. Ramage G, Bachmann S, Patterson TF, Wickes BL, Lopez-RibotJL. Investigation of multidrug effluxpumps in relation to fluconazole resistance in Candida albicans biofilms. J Antimicrob Chemother. 2002;49:973–980.
10. Kanafani ZA, Perfect JR. Antimicrobial resistance: Resistance to antifungal agents: mechanisms andclinical impact. Clin Infect Dis 2008;46:120–128.
11. Pappas, PG, Lionakis, MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nat Rev Dis Primers 2018; 4:18026.
12. Hospenthal DR, Murray CK, Rinaldi MG. The role of antifungal susceptibility testing in the therapy of candidiasis. Diagn Microbiol Infect Dis. 2004;48,153–160.
13. Alastruey-Izquıerdo A, Melhem MSC, Bonfıettı LX, Rodrıguez-Tudela JL Susceptibility test for fungi: Clinical and laboratorial correlations in medical mycology. Rev Inst Med Trop Sao Paulo 2015;57(19):57-64.
14. Orasch C, Marchetti O, Garbino J, Schrenzel J, Zimmerli S, Mühlethaler K et al. Candida species distribution and antifungal susceptibility testing according to European Committee on Antimicrobial Susceptibility Testing and new vs. old Clinical and Laboratory Standards Institute clinical breakpoints: A 6-year prospective candidaemia survey from the fungal infection network of Switzerland. Clin Microbiol Infect 2014;20:698–705.
15. Lindberg E, Hammarström H, Ataollahy, Kondori N. Species distribution and antifungal drug susceptibilities of yeasts isolated from the blood samples of patients with candidemia. Scientific Reports 2019;9:3838.
16. Dalyan Cilo B, Topaç T, Ağca H, Sağlam S, Efe K, Ener B. Comparison of Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution methods for determining the susceptibilities of Candida isolates. Bulletin of Microbiology 2018;52 (1):35-48.
17. Gülat S, Doluca Dereli M. Investigation of the expression levels of efflux pumps in fluconazole-resistant Candida albicans isolates. Bulletin of Microbiology 2014;48(2):325-334.
18. Coşkun Ö, Beşirbellioğlu B, Yıldıran ŞT, Gönlüm A, Pahsa A. In-vitro susceptibilities of Candida species isolated from candidemic patients against amphotericin B and fluconazole. Bulletin of Microbiology 2001;35:565-571.
19. Hope W W, Billaud EM, Lestner J, Denning DW. Therapeutic drug monitoring for triazoles. Curr Opin Infect Dis 2008; 21:580-586.