In Vitro Susceptibility and Trailing Growth Effect of Clinical Isolates of Candida Species to Azole Drugs

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Background: Emergence of resistance to respective antifungal drugs is a primary concern for the treatment of candidiasis. Hence, determining antifungal susceptibility of the isolated yeasts is of special importance for effective therapy. For this purpose, the clinical laboratory standard institute (CLSI) has introduced a broth microdilution method to determine minimum inhibitory concentration (MIC). However, the so-called “Trailing effect” phenomenon might sometimes pose ambiguity in the interpretation of the results.

Objectives: The present study aimed to determine the in vitro susceptibility of clinical isolates of Candida against azoles and the frequency of the Trailing effect.

Materials and Methods: A total of 193 Candida isolates were prospectively collected and identified through the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Using a broth microdilution test, according to the guidelines of CLSI M27-A3, antifungal susceptibilities of the isolated yeasts against Fluconazole (FLU), Itraconazole (ITR), Ketoconazole (KET) and Voriconazole (VOR) were assessed. Moreover, trailing growth was determined when a susceptible MIC was incubated for 24 hours, and turned into a resistant one after 48 hours of incubation.

Results: Among the tested antifungal drugs in this study, the highest rate of resistance was observed against ITR (28.5%) followed by VOR (26.4%), FLU (20.8%) and KET (1.5%). The trailing effect was induced in 27 isolates (14.0%) by VOR, in 26 isolates (13.5%) by ITR, in 24 isolates (12.4%) by FLU, and in 19 isolates (9.8%) by KET.

Conclusions: The monitoring of antifungal susceptibilities of Candida species isolated from clinical sources is highly recommended for the efficient management of patients. Moreover, the trailing effect should be taken into consideration once the interpretation of the results is intended.

Keywords: Antifungal Susceptibility, Trailing Effect, Candida

1. Background

Candidiasis is an infection with a wide scope of symptoms ranging from mild dermatosis to systemic infection with high mortality rate (1, 2). The yeasts belonging to the genus Candida are considered as the most frequent fungi isolated from cancer patients (3), the second cause of catheter-associated urinary tract infections, the third pathogenic organisms responsible for pediatrics sepsis (4, 5), and finally, the fourth cause of hospital-acquired fungemia with distinguished mortality rate (6). Recently, a significant switching in the type of the isolated species to non-albicans has been found (7, 8). Although Candida albicans is still isolated from clinical specimens as the main species, frequency of non-albicans species has considerably increased (7, 9, 10). At the same time, emerge of resistance of Candida species, in particular non-albicans isolates, to current antifungal drugs, is another current universal crisis (10-12). These resistant strains, especially among non-albicans species, increase treatment failure and risk of mortality. Also, they may be associated with patients’ prolonged hospital stays and sometimes, contribute to further complications (13). Several factors, including human practices, either overuse or abuse of antibiotics, and increase in the population of immune compromised patients, may contribute to the rise of this problem (13). Therefore, the first step to overcome antifungal resistance is tracking the resistance data locally.

In such regard, the two well-recognized standard antifungal susceptibility methods are those recommended by the clinical laboratory standard institute (CLSI) and the European committee on antimicrobial susceptibility commercial usages, provided the original work is properly cited.
testing (EUCAST) (14-16), but differ from one another in inoculation density and glucose content of the base media (14-18). Reading the plates and interpreting the results are major problems on the way to determine the minimum inhibitory concentration (MIC) by these methods. An important issue in the interpretation of the MIC results is a phenomenon known as trailing effect, which is the reduced but persistent growth of yeasts through the serial micro-dilution method (19). This phenomenon could complicate the interpretation of the results. Also, its frequency might be underestimated as it might be ignored while reading the plates (19).

Although the precise cause of this effect is far from being fully understood, a number of studies have proposed that up-regulation of some genes involved in the resistance to azole drugs, such as encoding lanosterol demethylase (ERG5), squalene epoxidase (ERG1) or efflux transporters, might have a role (20). Moreover, some authors have reported that the inoculum size (21), the incubation temperature (22), and strain-molecular characteristics (23) might also be involved. Others have reported adding glucose to RPMI (24) or adjustment of the medium pH ≤ 5 might suppress the trailing effect (25). Although the determination of MIC might be complicated by the trailing effect, it does not indicate clinical resistance. Based on a murine model (26), the isolates exhibiting the trailing effect in vitro, should be classed as susceptible strains in vivo.

Moreover, it has been previously shown that oropharyngeal candidiasis caused by multiple species. The yeasts were identified by the polymorphism (PCR-RFLP), as described by Mirhendi et al. (28, 29). In order to identify the species, total DNA was extracted via the glass bead method and purified by phenol-chloroform-isoamyl alcohol (25:24:1) (30). A pair of universal primers (ITS1, 5-TCCGTAGGTTGAACCTGCGG and ITS4, 5-TCCTCCGCTTATTGATATGC) (Metabion International, Martinsried, Germany) (28) was used to allow the amplification of the target ITS1-5.8s-ITS2 ribosomal DNA. Polymerase chain reaction amplification was carried out in a final volume of 50 µL. Each reaction contained 1 µL of template DNA, 0.5 µM of each primer, 0.20 mM of each deoxynucleoside triphosphate (dNTPs), 5 µL of 10 × PCR buffer, and 1.25 unite of Taq-DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany). The amplification protocol consisted of an initial denaturation step (94°C for five minutes), 35 cycles of denaturation (94°C for 30 seconds), annealing (56°C for 45 seconds), and extension (72°C for one minute), plus one cycle of final extension (72°C for seven minutes).

The amplified target sequence was digested with MspI (Roche Molecular Biochemicals, Mannheim, Germany) restriction endonuclease to yield the best species-specific pattern (28). Moreover, C. dubliniensis was differentiated from C. albicans using additional enzyme, (Bln1 (AvrII)) (Roche Molecular Biochemicals, Mannheim, Germany) (29). The digestion was performed by incubating the amplified PCR product with 10 U of the enzyme at 37°C for 2.5 hours. The digested fragments were separated on 2% agarose gel by electrophoresis for about one hour at 100 V and visualized by staining with ethidium bromide under UV light.

3.2. Antifungal Susceptibility Testing

Susceptibility to fluconazole (FLU), ketoconazole (KET), voriconazole (VOR), and itraconazole (ITR) was tested through the broth micro-dilution technique based on document M27-A3 of the clinical and laboratory standards institute (CLSI) (14). All antifungal drugs were purchased from Sigma-Aldrich (St. Louis, USA).

Concisely speaking, for the determination of antifungal activities against yeast, serial dilutions of the selected antifungals (Sigma-Aldrich, USA) (0.012 to 128 µg/mL) were prepared in 96-well microtitre plates using RPMI-1640 media (Sigma, St. Louis, USA) buffered with MOPS (Sigma, St. Louis, USA). The examined yeasts were suspended in 5 mL sterile 0.85% NaCl, and the densities were adjusted to 0.5 McFarland standards at 530 nm wavelengths by a spectrophotometer to yields stock suspension of 1 × 10^6 cells/mL.

Working suspension was prepared by making a 1/1000 dilution of the stock in buffered RPMI. Afterwards, 0.1 mL of the working inoculums was added to the wells except the 1st column and the plates were incubated at 32°C in a humid chamber. Growth controls consisting of RPMI-1640 medium and RPMI-1640 with 1% (v/v) DMSO were included for each tested isolate. In addition, 200 µL of un-inoculated, RPMI-1640 medium
was included as a sterility control and blank. The MICs were visually determined after 24 hours and 48 hours, respectively. The MICs were defined as the lowest drug concentration at which a predominant decrease in turbidity (approximately 50% and 90% inhibition) was observed, compared with that of the drug-free growth control well. Each experiment was performed in triplicates. The trailing effect, characterized by incomplete growth inhibition, was recorded after 48 hours of incubation. Moreover, 10 μL of media from the wells showing no visible growth were further cultured onto Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) plates to determine the Minimum fungicidal concentrations (MFCs). The MFC was determined as the lowest concentration that yielded ≤ 4 colonies, which corresponded to a mortality of 98% of the yeasts in the initial inoculum.

The interpretive criteria (breakpoints) used for determining the susceptibility to the examined antifungal drugs were similar to those proposed by CLSI (14). The MIC breakpoints for FLU of ≤ 8 μg/mL, 16 to 32 μg/mL and ≥ 64 μg/mL were used to respectively characterize the susceptible (S), susceptible dependent upon dose (SDD), and resistant (R) categories. For ITR, MIC breakpoints of ≤ 0.12μg/mL, 0.25 to 0.5 μg/mL and ≥ 1μg/mL were respectively considered S, SDD and R. Two quality control strains including C. albicans ATCC 10261, and C. krusei ATCC 6258 were used to validate the results. The examined yeasts were considered as having trailing phenomenon if they exhibited a susceptible MIC after 24 hours of incubation and a resistant MIC following 48 hours of incubation.

### 4. Results

The yeasts were identified by PCR-RFLP as follows: 114 C. albicans (59.1%), 27 C. parapsilosis (14.0%), 17 C. tropicalis (8.8%), 16 C. glabrata (8.3%), and 14 C. dubliniensis (7.2%), 3 C. krusei (1.6%) and 2 C. guilliermondii (1.0%). The antifungal susceptibilities to FLU, KET, VOR and ITR are summarized in Table 1. Seventy-three percent of the isolates were susceptible to FLU (MIC ≤ 16.0 μg/mL), 6.2% were susceptible dependent upon dose and the rest (20.8%) were resistant. Among 40 resistant isolates, 24 (60.0%) were identified as C. albicans, 11 (27.5%) as C. tropicalis, and three (7.5%) as C. dubliniensis. However, among each isolated species, C. tropicalis (64.7%) exhibited the highest rate of resistance to FLU followed in frequency by C. krusei (33.3%), C. dubliniensis (21.4%), C. albicans (21.0%) and C. parapsilosis (3.7%).

Of the examined isolates, 59.1% were susceptible to ITR, 12.4% were SDD and 28.5% were resistant. Of the 55 resistant isolates, 40 (72.7%) were identified as C. albicans, 11 (20%) as C. tropicalis, three (5.5%) as C. dubliniensis, and one (1.8%) as C. parapsilosis. Among the examined species, C. tropicalis with a frequency of 64.7% showed the highest rate of resistance to FLU followed by C. albicans, C. dubliniensis and C. parapsilosis with frequencies of 35.5%, 24.4% and 3.7%, respectively. All tested isolates of C. glabrata, C. guilliermondii, and C. krusei were susceptible to ITR.

Voriconazole susceptibility and resistance were observed for 73.6% and 26.4% of the Candida species, respectively. Of the tested species, C. tropicalis with a frequency of 47.1% was found to have the highest rate of resistant isolates followed by C. albicans (33.6%), C. dubliniensis (28.6%) and C. parapsilosis (3.7%). All examined isolates of C. glabrata, C. guilliermondii and C. krusei were proved to be susceptible to VOR. Of the examined isolates, only three yeasts were resistant to KET including two C. albicans and one C. dubliniensis. However, SDD to KET was found in 24 yeasts, including 21 C. albicans, two C. tropicalis, and one C. dubliniensis.

The distribution of the trailing effect frequencies in tested yeasts against theazole drugs is shown in Table 2. The highest rate of trailing effect was induced by VOR (14%), followed by ITR (13.5%), FLU (12.4%) and KET (9.8%).

### Table 1. Antifungal Susceptibilities of Clinical Candida Isolates Obtained by the Broth Microdilution Method

|                   | Fluconazole | Itraconazole | Ketoconazole | Voriconazole |
|-------------------|-------------|--------------|--------------|--------------|
|                    | R           | S            | SDD          | R            | S            | R            | S            |
| C. albicans       | 24 (60.0)   | 85 (60.4)    | 5 (4.6)      | 40 (72.7)    | 70 (61.4)    | 4 (16.6)     | 2 (66.7)     | 91 (54.9)    | 21 (87.5)    | 38 (74.5)    | 76 (51.6)    |
| C. tropicalis     | 11 (27.5)   | 4 (2.8)      | 2 (16.7)     | 11 (20.0)    | 5 (4.4)      | 1 (4.2)      | 0 (0.0)      | 15 (9.0)     | 2 (8.3)      | 8 (15.7)     | 9 (6.3)      |
| C. parapsilosis   | 1 (2.5)     | 25 (57.7)    | 1 (2.6)      | 11 (20.0)    | 5 (4.4)      | 1 (4.2)      | 0 (0.0)      | 15 (9.0)     | 2 (8.3)      | 8 (15.7)     | 9 (6.3)      |
| C. glabrata       | 0 (0.0)     | 14 (9.9)     | 2 (16.7)     | 0 (0.0)      | 8 (7.0)      | 8 (33.3)     | 0 (0.0)      | 16 (9.6)     | 0 (0.0)      | 0 (0.0)      | 16 (9.6)     |
| C. krusei         | 1 (2.5)     | 0 (0.0)      | 2 (16.7)     | 0 (0.0)      | 3 (2.6)      | 0 (0.0)      | 0 (0.0)      | 3 (1.8)      | 0 (0.0)      | 0 (0.0)      | 3 (2.1)      |
| C. dubliniensis   | 3 (7.5)     | 11 (27.8)    | 0 (0.0)      | 3 (5.5)      | 10 (8.8)     | 1 (4.2)      | 11 (33.3)    | 12 (7.2)     | 1 (4.2)      | 4 (7.8)      | 10 (7.0)     |
| C. guilliermondii | 0 (0.0)     | 2 (1.4)      | 0 (0.0)      | 0 (0.0)      | 1 (0.9)      | 1 (4.2)      | 0 (0.0)      | 2 (1.2)      | 0 (0.0)      | 0 (0.0)      | 2 (1.4)      |
| Total             | 40 (100.0)  | 141 (100.0)  | 12 (100.0)   | 55 (100.0)   | 114 (100.0)  | 24 (100.0)   | 3 (100.0)    | 166 (100.0)  | 24 (100.0)   | 51 (100.0)   | 142 (100.0)  |

Abbreviations: R, resistance; S, sensitive; SDD, susceptible dose dependent.

*Values are expressed as No. (%)*.  

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17 (89.5)
The Distribution of Frequencies of the Trailing Effect in Tested Yeasts Against the Azole Drugs

and
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isolates, 14.9% - 21% showed trailing growth in tests with
3 (11.1)
isolates ranging from 7% to 32% by FLU,
Azole Drugs


Itraconazole


Fluconazole is one of the most commonly prescribed antifungal agents for the treatment of mucocutaneous and systemic candidiasis. Resistance rate to this drug in different countries varies from 2.9% to 85%, which is due to the differences in treatment protocols and strategies (31-41). In the present study, about one fifth of the studied Candida species were resistant to FLU, which is at least twice as much as those previously reported (31, 36, 42, 43). Likewise, other recent studies conducted in Ahvaz, have reported a higher rate of resistance to FLU, among Candida species (40, 41). This variation in the pattern of susceptibilities to FLU suggests over prescription of thisazole due to its relatively low price and availability for both treatment and prophylaxes purposes. Among the tested Candida, C. tropicalis exhibited the highest rate of resistance to FLU, which is consistent with the finding of Seman et al. study (44). Although, C. glabrata has been known to be intrinsically resistant to FLU, similar to the findings of previous studies in Iran (31, 43), we found no FLU resistant strain within this species.

Itraconazole has a broader activity range than FLU and is usually prescribed for the treatment of systemic fungal infections including candidiasis. Recent studies have revealed that the rate of resistance to this drug varies from 2% to 38.4% (31-33, 36, 45-47). In the present study, among the tested azoles, the highest rate of resistance was found against ITR (28.5%), which was similar to some previous studies (31-33, 36, 45). Of the tested Candida species, C. albicans (72.7%) and C. tropicalis (20%) exhibited the highest rate of resistance to this azole.

Ketoconazole is an imidazole antifungal, which is usually prescribed topically as a shampoo or cream. The rates of resistance to KET among the Candida species remain low, with reported rates of 1% - 37% (31, 48). In this study, only 1.5% of the tested yeasts including two C. albicans and one C. dubliniensis were resistant to KET, which might be due to the mode of topical administration or lower prescription rate compared with other azoles.

It was previously reported that antifungal drugs are capable of inducing the trailing effect (19, 25, 49, 50). In another study, Ostrosky-Zeichner et al. (49) reported on the trailing effect in bloodstream Candida isolates ranging from 7% to 32% by FLU, ITR and VOR. In this study, C. albicans and C. dubliniensis exhibited the highest rate of trailing growth. Of the 114 C. albicans isolates, 14.9% - 21% showed trailing growth in tests with the examined azoles, which is consistent with what has been previously reported (49). However, none of the other four Candida species, including C. parapsilosis, C. krusei, C. guilliermondii and C. glabrata, were affected by this incident. As compared to other reports (19, 50), the lower rate of Trailing effect in this study may reflect differences in the type of specimens and the distribution of species. The impact of trailing effect on resistance has been previously studied (51), and does not apparently correlate with clinical outcome. In fact, this phenomenon might even elevate the 48-hour MICs of the drugs from one doubling dilution (for about 90% of isolates) to 16-fold higher compared to their corresponded 24-hour MICs (49, 52). Therefore, this effect should be taken into account when interpreting the results.

High resistance rate to ITR and FLU was found in this study, suggesting the need for regular investigation of the antifungal susceptibilities in medical centers for successfully treating the patients, in particular those with underlying diseases. These local antifungal susceptibility surveillances also help health policy makers to provide efficient guidelines for prophylaxis, empirical therapy, and for the management of candidiasis.

5. Discussion

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Table 2. The Distribution of Frequencies of the Trailing Effect in Tested Yeasts Against the Azole Drugs

| Species (Number) | Ketoconazole | Itraconazole | Fluconazole | Voriconazole |
|------------------|--------------|--------------|-------------|--------------|
|                  | TE | TE | TE | Te |
| C. albicans (114) | 17 (89.5) | 24 (92.3) | 22 (91.7) | 21 (77.8) |
| C. tropicalis (17) | 1 (5.3) | 0 (0.0) | 0 (0.0) | 3 (11.1) |
| C. parapsilosis (27) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| C. glabrata (16) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| C. krusei (3) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| C. dubliniensis (14) | 1 (5.3) | 2 (7.7) | 2 (8.3) | 3 (11.1) |
| C. guilliermondii (2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Total (193) | 19 (100.0) | 26 (100.0) | 24 (100.0) | 27 (100.0) |

Abbreviation: TE, Trailing Effect.
Values are expressed as No. (%).
Footnotes

Authors’ Contribution: Study concept and design: Kamiar Zomorodian; sample collection and laboratory examinations: Azadeh Bandegani, Navab Alinejad and Ali Poostforosh Fard; data interpretation: Keyvan Pakshir and Kamiar Zomorodian; drafting of the manuscript: Kamiar Zomorodian, Keyvan Pakshir and Ali Poostforosh Fard; manuscript revision: all authors; statistical analysis: Kamiar Zomorodian and Ali Poostforosh Fard.

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References

1. Zomorodian K, Haghighi NN, Rajaei N, Pakshir K, Tarazooie B, Vojdani M, et al. Assessment of Candida species colonization and denture-related stomatitis in complete denture wearers. Med Mycol. 2011;49(6):2208-11. doi: 10.1080/13693786.2010.507605. [PubMed: 20795762]

2. Danke L, Lazerza Mdos S, Nucci M. Fungal infections in the immunocompromised host. Mem Inst Oswaldo Cruz. 2000;95 Suppl 1:E5-8. [PubMed: 11424407]

3. Schelze S, Abdallah S, Gray G, Stubbings H, Gow I, Baker P, et al. Epidemiology of oral yeast colonization and infection in patients with hematological malignancies, head neck and solid tumors. J Oral Pathol Med. 2011;40(1):38-9. doi: 10.1111/j.1600-0714.2010.00937.x. [PubMed: 20923440]

4. Alangaden GJ. Nosocomial fungal infections: epidemiology, infection control, and prevention. Infect Dis Clin North Am. 2012;26(1):201-25. doi: 10.1016/j.idc.2011.10.003. [PubMed: 22360010]

5. Brissaud O, Guichoux J, Harambat J, Tandonnet O, Zaoutis T. Invasive fungal disease in PUCI: epidemiology and risk factors. Ann Intensive Care. 2012;2(1):6. doi: 10.1186/2110-5820-2-6. [PubMed: 22356688]

6. Arendrup MC. Epidemiology of invasive candidiasis. Curr Opin Crit Care. 2000;6(3):445-52. doi: 10.1097/00001088-200006000-00004. [PubMed: 10710765]

7. Tan TY, Tan AT, Tee NW, Ng IS, Chee CW. The increased role of non-albicans species in candidaemia: results from a 3-year surveillance study. Mycoses. 2010;53(8):315-21. doi: 10.1111/j.1437-093X.2010.01956.x. [PubMed: 19602626]

8. Farooqi IQ, Jabeen K, Saeed N, Iqbal N, Malik B, Lockhart SR, et al. Invasive candidiasis in Pakistan: clinical characteristics, species distribution and antifungal susceptibility. J Med Microbiol. 2013;62(7):259-68. doi: 10.1099/jmm.0.048785-0. [PubMed: 23105261]

9. Otero JR, Wattal C, Goel N, Raveendran R, Datta S, Prasad K. Antifungal susceptibility testing in vitro and therapeutic outcome in vivo for fluconazole susceptibility testing of Candida spp. J Clin Microbiol. 2010;48(5):1592-9. doi: 10.1128/JCM.01445-09. [PubMed: 20354424]

10. Arendrup MC, Diekema DJ. Progress in antifungal susceptibility testing of Candida spp. J Clin Microbiol. 2008;46(10):2985-7. doi: 10.1128/JCM.00787-08. [PubMed: 18828859]

11. Pfaller MA, Castanheira M, Diekema DJ, Messer SA, Moet GJ, Jones RN. Comparison of European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Etest methods with the CLSI broth microdilution method for echinocandin susceptibility testing of Candida species. J Clin Microbiol. 2010;48(5):1592-9. doi: 10.1128/JCM.01445-09. [PubMed: 20354424]

12. Arendrup MC, Diekema DJ. Progress in antifungal susceptibility testing of Candida spp. by use of Clinical and Laboratory Standards Institute broth microdilution methods, 2010 to 2012. J Clin Microbiol. 2012;50(9):2846-56. doi: 10.1128/JCM.00973-12. [PubMed: 22740712]

13. Coenye T, De Vos M, Vandenbossche D, Neils H. Factors influencing the trailing endpoint observed in Candida albicans susceptibility testing using the CLSI procedure. Clin Microbiol Infect. 2008;14(5):495-7. doi: 10.1111/j.1600-2689.2008.00958.x. [PubMed: 18341604]

14. Lee MK, Williams LE, Warnock DW, Arthington-Skags BA. Drug resistance genes and trailing growth in Candida albicans isolates. J Antimicrob Chemother. 2004;53(3):237-44. doi: 10.1093/jac/dkt040. [PubMed: 14688046]

15. Cuenca-Estrella M, Diaz-Guerra TM, Mellado E, Rodrigue-Tudela L. Influence of glucose supplementation and inoculum size on growth kinetics and antifungal susceptibility testing of Candida spp. J Clin Microbiol. 2001;39(2):529-32. doi: 10.1128/JCM.39.2.529-532.2001. [PubMed: 11058100]

16. Agarwal D, Patterson TF, Rinaldi MG, Revankar SG. Trailing-end-point phenotype of Candida spp. in antifungal susceptibility testing to fluconazole is eliminated by altering incubation temperature. J Med Microbiol. 2007;56(Pt 7):703-4. doi: 10.1099/jmm.0.047680-0. [PubMed: 17577072]

17. Braga-Silva LA, Mesquita DG, Ribeiro MD, Carvalho SM, Fracalanza SE, Santos AL. Trailing-end-point phenotype antibiotic-sensitive strains of Candida albicans produce different amounts of aspartyl peptide. Braz J Med Biol Res. 2009;42(8):765-70. [PubMed: 19649403]

18. Rodríguez-Tudela JL, Martínez-Suárez J. Improved medium for fluconazole susceptibility testing of Candida albicans. Antimicrob Agents Chemother. 1994;38(6):1045-8. [PubMed: 8145758]

19. Marr KA, Rustad TR, Rex JH, White TC. The trailing end point phenotype in antifungal susceptibility testing is pH dependent. Antimicrob Agents Chemother. 1999;43(5):1383-6. doi: 10.1128/AAC.39.5.1383-1386.1999. [PubMed: 10348757]

20. Rex JH, Nelson PW, Paetznick VS, Lozano-Chiu M, Espinel-Ingroff A, Naissir EJ. Optimizing the correlation between results of testing in vitro and therapeutic outcome in vivo for fluconazole by testing critical isolates in a murine model of invasive candidiasis. Antimicrob Agents Chemother. 1998;42(4):1299-34. [PubMed: 9449272]

21. Revankar SG, Kirkpatrick WR, McAtee RK, Fothergill AW, Redding SW, Rinaldi MG, et al. Interpretation of trailing endpoints in antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards method. J Clin Microbiol. 1998;36(5):1353-6. doi: 10.1128/JCM.36.5.1353-1356.1998. [PubMed: 9439393]

22. Mirhendi H, Makimura K, Khoramizadeh M, Yamaguchi H. A One-Enzyme PCR-RFLP Assay for Identification of Six Medically Important Candida Species. Nippon Ishinkin Gakkai Zasshi. 2006;47(3):225-9. doi: 10.3314/jmm.47.225. [PubMed: 16680456]

23. Mirhendi H, Makimura K, Zomorodian K, Maeda N, Oshima T, Yamaguchi H. Differentiation of Candida albicans and Candida
Identification and In-Vitro Susceptibility of Candida albicans and C. dubliniensis Isolated from Immu-nocompromised Patients. IR-CMJ. 2009; 4(1):391-7.

30. Yamada Y, Makimori K, Merhendi H, Ueda K, Nishiyama Y, Yama-
guchi H, et al. Comparison of different methods for extraction of mitochondrial DNA from human pathogenic yeasts. Jpn J Infect Dis. 2002;55(4):322-5. [PubMed: 12403909].

31. Badiei P, Alborzi A, Davarpanah MA, Shakiba E. Distributions and antifungal susceptibility of Candida species from mucosal sites in HIV positive patients. Arch Iran Med. 2010;13(4):282-7. [PubMed: 20505766].

32. Panizo MM, Reviakina V, Dolande M, Selgrad S. Candida spp. in vitro susceptibility profile to four antifungal agents. Resistance surveil-

lance study in Venezuelan strains. Med Mycol. 2009;47(2):337-43. doi:10.1080/13693780802444339. [PubMed: 18651808].

33. Lee JS, Shin JH, Lee K, Kim MN, Shin BM, Uh Y, et al. Species dis-

tribution and susceptibility to azole antifungals of Candida bloodstream isolates from eight university hospitals in Korea. Yonsei Med J. 2007;48(5):779-86. doi: 10.3349/ymj.2007.48.5.779. [PubMed: 17963334].

34. Silva V, Alvarado D, Diaz MC. Antifungal susceptibility of 50 Candida isolates from invasive mycoses in Chile. Med Mycol. 2004;42(2):283-9. [PubMed: 15282244].

35. Citiak S, Ozcelik B, Cesur S, Abbasoglu U. In vitro susceptibility of Candida species isolated from blood culture to some antifungal agents. Jundishapur J Microbiol. 2011;4(2):509-26.

36. Fleck R, Dietz A, Hof H. In vitro susceptibility of Candida species to five antifungal agents in a German university hospital as se-

sessed by the reference broth microdilution method and Etest. J Antimicrob Chemother. 2005;56(4):767-71. doi:10.1093/jac/dkl555. [PubMed: 17293869].

37. Koptaw A, Biswas D, Sharma P, Gupta A, Jindal P. An observa-
tional study on the epidemiological and mycological profile of Candidemia in ICU patients. Med Sci Mont. 2011;47(6):CR663-8. [PubMed: 22037747].

38. Mulu A, Kassu A, Anagaw B, Moges B, Gelaw A, Alemayehu M, et al. Frequent detection of ‘azole’ resistant Candida species among late presenting AIDS patients in northwest Ethiopia. BMC Infect Dis. 2013;13:82. doi:10.1186/1471-2148-13-82. [PubMed: 23987813].

39. Zarei Mahmoudabadi A, Zarrin M, Behsheti Fard M. Antifungal susceptibility of Candida species isolated from candiduria. Jundishap-

ur J Microbiol. 2012;6(1):24-8. doi:10.5821/jmm.4633.

40. Saleheiz Z, Seifi Z, Mahmoudabadi A. Sensitivity of Vaginal Isolates of Candida to Eight Antifungal Drugs Isolated From Ahvaz, Iran. Jundishapur J Microbiol. 2012;6(4):574-7. doi:10.5821/jmm.4536.

41. Parisa R, Alborzi A, Shakiba E, Ziyaeyan M, Rasuli M. Molecular Identification and In-Vitro Susceptibility of Candida albicans and Candida dubliniensis using a single-enzyme PCR-RFLP method. Jpn J Infect Dis. 2005;58(4):235-7. [PubMed: 16162589].

42. Khosravi AR, Shokrhi H, Mansouri P, Khatrane F, Zilgari T. Candida species isolated from nails and their in vitro susceptibility to antifungal drugs in the department of Dermatology (University of Tehran, Iran.). Med J Mycol. 2008;28(4):210-5. doi:10.1016/j.aacm.

43. Seman MS, Ramli RB, khatir TM. Antifungal susceptibility patterns among Candida species isolated from blood at Univer-
siti Kebangsaan Malaysia Medical Centre. Sains Malaysia. 2012;41(8):360-7.

44. Metin DY, Hilmiligou-Polat S, Samioglu P, Doganay-olfazoglu B, Inci R, Tumbay E. Evaluation of antifungal susceptibility testing with microdilution and Etest methods of Candida blood isolates. Mycopathologia. 2011;172(3):387-99. doi: 10.1007/s11046-011-9413-9. [PubMed: 21424603].

45. Badiei P, Alborzi A. Susceptibility of clinical Candida species isolates to antifungal agents by Etest, Southern Iran: A five year study. Iran J Microbiol. 2011;3(4):183-8. [PubMed: 22530086].

46. Peman J, Canton E, Quindos G, Eraso E, Alcobia J, Guineu J, et al. Epidemiology, species distribution and in vitro antifungal sus-

ceptibility of fungaemia in a Spanish multicentre prospective survey. J Antimicrob Chemother. 2012;67(5):1381-7. doi:10.1093/ijac/dzs019. [PubMed: 22353681].

47. Taura DW, Majie MH, Koki AM, Musa MG. Antifungal Resistance Among Candida species From Patients with Genitourinary Tract Infection at Muhammad Abdullahi Wase Specialist Hospital, Kano - Nigeria. Nigerian J Basic Appl Sci. 2011;1(1). doi:10.4314/njbai.v1i1.s.1.

48. Ostrosky-Zeichner L, Rex JH, Pappas PG, Hamill RJ, Larsen RA, Horowitz HW, et al. Antifungal susceptibility survey of 2,000 bloodstream Candida isolates in the United States. Antimicrob Agents Chemother. 2003;47(10):3449-54. [PubMed: 14506023].

49. Girmenia C, Tuccinardi C, Santilli S, Mondello F, Monaco M, Cash-

one A, et al. In vitro activity of fluconazole and voriconazole against isolates of Candida albicans from patients with haematological malignancies. J Antimicrob Chemother. 2000;46(4):479-83. [PubMed: 10910079].

50. Arthington-Skaggs BA, Lee-Yang W, Ciblak MA, Frade JP, Brandt ME, Hajieh RA, et al. Comparison of visual and spectrophotome-

tric methods of broth microdilution MIC end point determina-

tion and evaluation of a sterol quantitation method for in vitro susceptibility testing of fluconazole and itraconazole against Candida dubliniensis using a single-enzyme PCR-RFLP method. Jundishapur J Microbiol. 2016;9(2):e28666.