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

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Abstract

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

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 those encoding lanosterol demethylase (ERG11), 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 $\leq$ 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 trailing isolates respond well to a low dose of fluconazole (27). In the present study, we examined the *in vitro* activity of azole drugs against clinical isolates of *Candida* species by broth micro-dilution. The frequency of trailing effect in this method was examined, as well.

2. 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.

3. Materials and Methods

3.1. Isolation and Identification of the Species

A total of 193 *Candida* strains, isolated from clinical specimens at Shiraz University of Medical Sciences and Tehran University of Medical Sciences, were tested. Isolates were obtained from different sites of the body including the oral cavity (n = 118, 57.6%), blood (n = 64, 30.7%), genital tract (n = 17, 8.3%) and respiratory tract (n = 7, 3.4%). All isolated yeasts were cultured on CHROMagar *Candida* (CHROMagar Microbiology, Paris, France) for primary identification and isolation of mixed species. The yeasts were identified by the polymerase chain reaction- restriction fragment length 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-TCCGTAGGTGAACCTGCGG 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. (B1nI (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 $1 - 5 \times 10^6$ 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. krus*ei (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 ITR followed by *C. albicans*, *C. dubliniensis* and *C. parapsilosis* with frequencies of 35.1%, 21.4% 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* isolates, 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 the azole 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[a]

	Fluconazole			Itraconazole			Ketoconazole			Voriconazole	
	R	S	SDD	R	S	SDD	R	S	SDD	R	S
C. albicans	24 (60.0)	85 (60.4)	5 (41.6)	40 (72.7)	70 (61.4)	4 (16.6)	2 (66.7)	91 (54.9)	21 (87.5)	38 (74.5)	76 (53.6)
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 (17.7)	1 (8.3)	1 (1.8)	17 (14.9)	9 (37.5)	0 (0.0)	27 (16.3)	0 (0.0)	1 (2.0)	26 (18.3)
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 (11.3)
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 (7.8)	0 (0.0)	3 (5.5)	10 (8.8)	1 (4.2)	1 (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, sensitive dose dependent.
[a]Values are expressed as No. (%).

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

Species (Number)	Azole Drugs			
	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.
[a]Values are expressed as No. (%).

5. Discussion

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 this azole 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.

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Footnotes

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

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