In Vitro Susceptibilities of *Candida albicans* Isolates to Antifungal Agents in Tokat, Turkey

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Background: *Candida albicans* is the pathogenic species most commonly isolated from fungal infections. Management of these infections depends on the immune status of the host, severity of disease, and the choice of antifungal drug. In spite of the development of new antifungal drugs, epidemiological studies have shown that resistance to antifungal drugs in *C. albicans* strains is becoming a serious problem.

Objectives: The aim of this study was to evaluate the in vitro susceptibility of *C. albicans* isolates to ketoconazole, fluconazole, itraconazole, voriconazole, posaconazole, amphotericin B, caspofungin, and anidulafungin.

Materials and Methods: A total of 201 *C. albicans* isolates were collected from clinical specimens. Antifungal susceptibility tests were performed using the Etest.

Results: All the tested *C. albicans* isolates were found to be susceptible to amphotericin B and anidulafungin. Although none of the isolates showed resistance to caspofungin, 15% of the isolates were classified as showing intermediate resistance. The resistance rates of *C. albicans* isolates to ketoconazole, fluconazole, itraconazole, voriconazole and posaconazole were 32%, 34%, 21%, 14% and 14%, respectively.

Conclusions: Our findings indicate that resistance of *C. albicans* strains to azoles is more common in Tokat, Turkey. Therefore, a strategy to control the inappropriate and widespread use of antifungal drugs is urgently needed. Fungal culturing and antifungal susceptibility testing will be useful in patient management as well as resistance surveillance.

Keywords: Amphotericin B; Azoles; Echinocandins; Antifungal Drug Resistance; *Candida albicans*

1. Background

In the past few decades, the widespread use of broad-spectrum antibiotics, corticosteroids, immunosuppressants, and antineoplastic agents has given rise to an increase in fungal infections (1-3). *Candida albicans* is the pathogenic species most commonly isolated from these fungal infections (4). The management of these infections depends on the immune status of the host, severity of disease, and the choice of antifungal drug (5). Recently, new antifungal drugs have been developed and introduced into clinical use for the treatment of fungal infections. In spite of the development of new antifungal drugs, epidemiological studies have shown that resistance to antifungal drugs in *C. albicans* strains is becoming a serious problem (6, 7).

Triazoles are the most widely used antifungal drugs (3). However, increased use of triazoles in both prophylactic and empiric therapy has resulted in the development of azole resistance in *Candida* species (6). Many studies have reported increased fluconazole resistance rates in *C. albicans* isolates (8, 9). Voriconazole and posaconazole, which are newer triazoles, have broad-spectrum activity against yeasts and molds, including fluconazole-resistant *Candida* spp. (10, 11). Although voriconazole and posaconazole are active against fluconazole-resistant *Candida* spp., cross-resistance has been reported (11, 12).

The mode of action of echinocandins such as caspofungin and anidulafungin is different from that of azole drugs. Echinocandins act by inhibiting 1,3-β-glucan synthesis in fungal cells (13). They have fungicidal activity against *Candida* spp., including those that are resistant to other antifungal agents (14-16). On the other hand, the acquisition of resistance to caspofungin has been observed (17). *In vitro* antifungal susceptibility testing is an important tool for the selection of a proper antifungal therapy, since increasing antifungal resistance rates in *C. albicans* strains and treatment failure have been reported frequently (8, 9, 17, 18). Antifungal susceptibility testing also enables a characterization of the changes in antifungal sensitivity patterns of *C. albicans* strains. The agar-based Etest is a useful method for determining in vitro susceptibilities of *Candida* spp. to the azoles, amphotericin B and caspofungin (19-22).

2. Objectives

The aim of this study was to evaluate the in vitro susceptibility of *C. albicans* isolates to fluconazole, itraconazole, ketoconazole, voriconazole, posaconazole, amphotericin B, caspofungin, and anidulafungin using the Etest.

3. Materials and Methods

A total of 201 *C. albicans* isolates were collected from clinical specimens submitted to the mycological laboratory of the clinical microbiology department, Gaziosmanpasa university hospital, Tokat, Turkey, between May 2007 and January 2012. *Candida albicans* isolates were identified by a germ tube test, in which chlamydospore formation on cornmeal agar plus Tween 80 (23) was assayed using an API 20C AUX Commercial System (BioMeriux, Marcy-l' Etoile, France). Isolates were stored in 20% glycerol at -80°C until use.

Antifungal susceptibility tests were performed using amphotericin B, voriconazole, caspofungin (AB Biodisk, Solna, Sweden), fluconazole, ketoconazole, itraconazole, posaconazole, and anidulafungin (Liofilchem, Teramo, Italy) Etest strips. Etest strips were stored at -20°C until use. The Etest was performed in accordance with the manufacturer's instructions. The agar plates were prepared using RPMI-1640 medium (Sigma, St. Louis, USA) supplemented with 1.5% agar and 2% glucose and buffered to a pH of 7.0 with 0.165 mol L^{-1} MOPS (3-[N-morpholino] propanesulfonic acid) (Sigma, St. Louis, USA). Yeast colonies were suspended in saline and the turbidity of the final inoculum was adjusted to 0.5 McFarland. The agar plates were inoculated by dipping a sterile swab into the suspension and swabbing the agar surface in three different directions. After the plates were allowed to dry in a safety cabinet for 15 min, Etest strips were applied on to the agar surface by using sterile forceps. The plates were incubated in ambient air or at 35°C for 24 - 48 hours. The minimum inhibitory concentration (MIC) was determined as 80% inhibition for the azoles and echinocandins and 100% inhibition for amphotericin B, and recorded as the drug concentration at the point where the ellipse intersected the MIC scale on the Etest strip. Quality control was performed using *Candida albicans* ATCC 90028. All tests were performed in duplicate.

Species-specific breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) M27-S4 document were used to evaluate the susceptibilities of isolates against voriconazole, fluconazole, itraconazole, caspofungin, and anidulafungin (24). These breakpoints are shown in Table 1. No interpretive criteria for posaconazole, ketoconazole, and amphotericin B are available in the CLSI M27-S4 document. Therefore, for amphotericin B and ketoconazole, MIC breakpoints recommended by previous researchers were used (25, 26), and voriconazole breakpoints were used for posaconazole. Isolates with MICs < 1 µg/mL for amphotericin B, ≤ 0.125 µg/mL for ketoconazole, and ≤ 0.125 µg/mL for posaconazole were considered as susceptible. Isolates with MICs from 0.25 µg/mL to 0.5 µg/mL for ketoconazole was considered as to be dose-dependently susceptible. Isolates with MICs ≤ 0.25 - 0.5 µg/mL for posaconazole were considered to show intermediate resistance. Isolates with MICs ≥ 2 µg/mL for amphotericin B, ≥ 1 µg/mL for ketoconazole, or ≥ 1 µg/mL for posaconazole were considered as resistant.

4. Results

The resistance rates, MIC ranges, MIC50 values, and MIC90 values of fluconazole, itraconazole, ketoconazole, voriconazole, posaconazole, amphotericin B, caspofungin, and anidulafungin for all the *C. albicans* isolates are summarized in Table 2.

Table 1. CLSI Breakpoints (BP) for *Candida albicans* (µg/mL)

C. albicans	Susceptible	Susceptible Dose-Dependent	Intermediate	Resistant
Fluconazole				
M27-A3 BP	≤ 8	16 - 32	-	≥ 64
M27-S4 BP	≤ 2	4	-	≥ 8
Itraconazole	≤ 0.12	0.25 - 0.5	-	≥ 1
Voriconazole				
M27-A3 BP	≤ 1	-	2	≥ 4
M27-S4 BP	≤ 0.12	-	0.25 - 0.5	≥ 1
Caspofungin				
M27-A3 BP	≤ 2	-	-	-
M27-S4 BP	≤ 0.25	-	0.5	≥ 1
Anidulafungin				
M27-A3 BP	≤ 2	-	-	-
M27-S4 BP	≤ 0.25	-	0.5	≥ 1

Table 2. *In Vitro* Activities of Fluconazole, Itraconazole, Ketoconazole, Voriconazole, Posaconazole, Amphotericin B, Caspofungin and Anidulafungin Against 201 *Candida albicans* Isolates

Antifungal Agents	MIC, μg/mL			Resistant [a]
	Range	**50%**	**90%**	
Fluconazole	0.064 - 256	1	> 256	69 (34)
Itraconazole	0.004 - 32	0.016	> 32	42 (21)
Ketoconazole	0.002 - 32	0.016	> 32	64 (32)
Voriconazole	0.002 - 32	0.012	> 32	29 (14)
Posaconazole	0.004 - 32	0.047	> 32	29 (14)
Amphotericin B	0.003 - 0.25	0.016	0.064	0 (0)
Anidulafungin	< 0.002 - 0.006	< 0.002	0.002	0 (0)
Caspofungin	0.012 - 0.5	0.19	0.38	0 (0)

[a] Data are presented as No. (%).

MIC values of 201 *C. albicans* strains were in the range of 0.003 - 0.25 μg/mL for amphotericin B. All tested *C. albicans* isolates were found to be susceptible to amphotericin B and anidulafungin. For anidulafungin, the MIC values ranged between < 0.002 μg/mL and 0.006 μg/mL and the majority of isolates (96 %) had MIC values < 0.002 μg/mL. On the other hand, for caspofungin, 15% of the isolates were determined to show intermediate resistance. The resistance rates of *C. albicans* isolates to ketoconazole, fluconazole, itraconazole, voriconazole and posaconazole were 32%, 34%, 21%, 14%, and 14%, respectively. All fluconazole resistant isolates had MIC values > 256 μg/mL. A total of 29 (14%) isolates were determined to be resistant to all tested azoles.

Only three (1.5%) *C. albicans* isolates were classified as dose-dependently susceptible to fluconazole according to revised CLSI breakpoints. Four isolates (2%) were found to be dose-dependently susceptible to itraconazole, whereas 2 isolates (1%) were dose-dependently susceptible to ketoconazole. None of the tested isolates was categorized to show intermediate resistance to voriconazole, although ten isolates (5%) were determined to show intermediate resistance to posaconazole. All the isolates that showed intermediate resistance to posaconazole were found to be susceptible to voriconazole.

5. Discussion

In this study, we found that all isolates were susceptible to amphotericin B. Similar results have been observed in previous studies in our country and the other European countries (27-30). Messer et al. have measured the MIC range for amphotericin B as 0.12 - 2 mg/L in an international surveillance study (31). On the other hand, Santhanam et al. (32) have documented amphotericin B MICs ranging from 0.25 to 16 mg/L in Malaysia. Recently, Badiee and Alborzi (12) have reported the resistance rate of *C. albicans* isolates to amphotericin B was 7% in Southern Iran.

In our study, 34% of the *C. albicans* isolates with MIC90 > 256 mg/L were found to be resistant to fluconazole.

Similarly, Zarei Mahmoudabadi et al. (33) showed that 55.2% of the *C. albicans* strains isolated from candiduria were resistant to fluconazole. In another study reported by the same authors, the resistance rate of *C. albicans* to fluconazole was 59.2% (34). In contrast to our findings, previous studies have reported low resistance rates for fluconazole (28, 30, 32, 35-38). The resistance rates to fluconazole, itraconazole, and voriconazole in our study were also higher than those reported in a previous study conducted in a region west of Turkey between 2008 and 2009 (27). Variation of these resistance rates may result from differences in the patient population, prior exposure to azoles, and different breakpoint values. It is important to emphasize that CLSI has recently established new species-specific MIC breakpoints to evaluate susceptibility to fluconazole, itraconazole, and voriconazole in *C. albicans* strains. Therefore, *C. albicans* isolates for which the fluconazole MIC was ≥ 8 mg/L were considered non-susceptible in this study; the MIC limit previously was ≥ 64 mg/L. Fothergill et al. (39) have evaluated the effect of new MIC breakpoints on azole and echinocandin resistance patterns in *Candida* species; the resistance rates in *C. albicans* isolates according to the new CLSI criteria were found to be higher than those determined previously.

Among the 69 fluconazole-resistant isolates, 38 (55%) were also resistant to both ketoconazole and itraconazole, 29 (42%) were resistant to ketoconazole, itraconazole, voriconazole, and posaconazole. Previous studies have documented that decreased susceptibility to fluconazole is associated with decreased susceptibility to other azoles (10, 35, 40, 41). Barchiesi et al. (42) have detected that the MICs of itraconazole for fluconazole-resistant *C. albicans* isolates were significantly higher than those for fluconazole-susceptible isolates, indicating cross-resistance between azoles. Numerous azole resistance mechanisms have been described, such as the induction of *CDR* and *MDR* genes-encoded efflux pumps, overexpression of 14-α demethylase, modification of the target enzyme structure, alteration of the ergosterol synthesis pathway,

reduction of fungal membrane permeability, etc. Induction of the *CDR* gene-encoded efflux pump and modification of target enzyme structure can result in triazole resistance in *C. albicans* strains, whereas induction of the *MDR* gene-encoded efflux pump is only responsible for fluconazole resistance (6). In our study, resistance rates to itraconazole among *C. albicans* strains were lower than those for ketoconazole and fluconazole. Cartledge et al. (40) have suggested that resistance associated with a reduction in fungal membrane permeability might result in resistance to ketoconazole and fluconazole rather than to itraconazole, because itraconazole is more lipid soluble than ketoconazole and fluconazole.

Many authors have documented that azole resistance in *Candida* strains has been associated with previous exposure to fluconazole (3, 5, 6, 38, 40). The widespread use of fluconazole, due to its relative safety and high oral bioavailability, for treatment and prophylaxis in our region may be the cause of the high azole resistance rates observed in our study. In addition, the availability of azole drugs without a prescription in our country may contribute to the development of azole resistance. All *C. albicans* isolates were susceptible to anidulafungin. Similar results have been reported by Fothergill et al. and Arendrup et al. (39, 43). In contrast to our findings, Faria-Ramos et al. (29) have documented the rate of anidulafungin resistance as 4% in *C. albicans* isolates. Resistance to caspofungin among *C. albicans* isolates has been reported by previous researchers (29, 43). Ghahri et al. (44) have observed that the MIC range for caspofungin as 0.125 - 4 mg/L in *Candida* species isolated from blood specimens. Although none of the isolates was found to be resistant to caspofungin, based on the new CLSI criteria, 15% of isolates were classified as showing intermediate resistance.

Our findings indicate that azole resistance in *C. albicans* strains is more common in our region. High azole resistance rates must be considered when selecting antifungal drugs for treatment or prophylaxis. Fungal culturing and antifungal susceptibility testing will be useful in patient management as well as resistance surveillance. We urgently need a strategy to control the inappropriate and widespread use of antifungal drugs. Application of antifungal control programs may contribute to prevent the increase of antifungal resistance.

Authors' Contributions

Study concept and design: Gulgun Yenişehirli, Yunus Bulut. Acquisition of data: Nermin Bulut. Analysis and interpretation of data: Aydan Yenisehirli. Drafting of the manuscript: Gulgun Yenisehirli. Critical revision of the manuscript for important intellectual content: Aydan Yenisehirli.

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