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In vitro susceptibility of seven antifungal agents against dermatophytes isolated in İstanbul

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Background/aim: Dermatophytes are the causative agents of dermatophytosis, which is a common disease worldwide that affects the hair, skin, and nails. Dermatophytes comprise more than 40 species in 3 genera: *Microsporum*, *Trichophyton*, and *Epidermaphyton*. In this study, we aimed to determine the effectiveness of seven antifungal agents: amphotericin B, terbinafine, itraconazole, voriconazole, ketoconazole, miconazole, and fluconazole.

Materials and methods: A sensitivity study was performed using a microdilution method in accordance with the CLSI M38-A2 standards using isolates of *Trichophyton rubrum* (n = 55), *Microsporum canis* (n = 9), and *Trichophyton interdigitale* (n = 2), which were identified by sequencing the internal transcribed spacer region of the rDNA.

Results: According to the results of antifungal sensitivity tests, the geometric mean (GM) minimum inhibitory concentration (MIC) against *T. rubrum* was 0.10 µg/mL for ketoconazole, 0.20 µg/mL for itraconazole, 0.07 µg/mL for miconazole, 0.48 µg/mL for fluconazole, 2.27 µg/mL for amphotericin B, 0.06 µg/mL for voriconazole, and 0.06 µg/mL for terbinafine.

Conclusion: The most effective antifungal drugs were voriconazole and terbinafine, both of which had a GM MIC of 0.06 µg/mL.

Key words: Antifungal, *Trichophyton rubrum*, dermatophytes, susceptibility

1. Introduction

Dermatophytes are keratinophilic hyaline molds that can cause disease in keratinized tissues like hair, skin, and nail. Depending on the reservoir and route of transmission, dermatophytes may be of anthropophilic (human), zoophilic (animals), or geophilic (soil) origin. Dermatophytes comprise more than 40 species in 3 genera: *Microsporum*, *Trichophyton*, and *Epidermaphyton*. The most common etiological agents are *T. rubrum*, *T. mentagraphytes*, *T. interdigitale*, *T. tonsurans*, and *Microsporum canis*. *T. rubrum* is the most frequently isolated agent in clinics (1). Dermatomycosis refers to diseases caused by yeasts and filamentous molds, whereas diseases caused by dermatophytes are called dermatophytosis (e.g., tinea, ringworm). Dermatophytes are the causative agents of the most common superficial fungal diseases in the world. These organisms are named according to the affected body site: *T. capitis* (head), *T. corporis* (trunk), *T. cruris* (perianal area), *T. pedis* (foot and interdigital area), and *T. unguium* (nail). Although the disease can affect individuals of all ages, *T. capitis* and *T. corporis* are more common among children, whereas *T. pedis* is more common among adults (2). The disease is more prevalent among individuals with diabetes and immune system disorders. Transmission can occur via direct contact with infected individuals as well as by sharing of household items such as brushes, shower facilities, carpeting, from household pets, and by autoinoculation (3).

Although the infection is neither painful nor life-threatening, accurate diagnosis and effective treatment are still important since the disease is widespread and contagious, causes aesthetic issues, and lowers life quality. Prolonged treatment may result in significant side effects of the drugs and treatment costs. In cases where infection is mild and localized, topical treatment is applied. Systemic treatment can be administered in chronic severe infections. Emergence of drug resistance, lack of compliance to treatment, and incorrect treatment may delay recovery and cause relapses. High relapse rates may be associated with circulatory impairment, drug interactions, advanced

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age, inadequate treatment, and lack of compliance (4,5). In Turkey, the presence of resistant isolates and poor patient compliance with treatment protocols are important issues resulting in treatment failure.

Selecting the appropriate antifungal agent is critical in order to prevent relapses and treatment failure, and to achieve complete recovery. Sensitivity tests enable monitoring for resistance development (6). Since diagnosis of the responsible species based on phenotypic features is difficult and leads to delays in treatment, molecular diagnosis is gaining importance (7).

In the present study, we aimed to determine the effectiveness of seven antifungal agents by following the CLSI M38-A2 (8) protocol, namely fluconazole (FLZ), ketoconazole (KCZ), miconazole (MCZ), voriconazole (VOR), terbinafine (TER), itraconazole (ITR), and amphotericin B (AMP B) against 66 dermatophyte strains that were diagnosed by DNA sequencing (9).

2. Materials and methods

2.1. Fungal isolates

The study included 66 strains isolated from clinical specimens of patients who were evaluated at the dermatology outpatient clinic of Cerrahpaşa University Faculty of Medicine and Şişli Etfal Hamidiye Training and Research Hospital and who were suspected to have dermatomycosis. Specimens were inoculated in SDA (Sigma-Aldrich, Madrid, Spain) growth medium for culture. Cultures yielding growth were evaluated based on colony morphology and microscopic properties. Diagnosis of the species was made by sequencing the internal transcribed spacer region of rDNA. The isolates were kept in a sterile saline solution and in petri dishes at +4 °C for later analysis.

2.2. Growth medium

Tests were performed using bicarbonate-free RPMI 1640 growth media (Sigma-Aldrich, Madrid, Spain) at pH 7, buffered with 0.165 M/L morpholino-propanesulfonic acid (MOPS) and containing L-glutamine and phenol red as pH indicator.

2.3. Inoculum preparation

In order to enhance pure culture and conidia growth, strains were re-inoculated in PDA (Oxoid, Basingstoke, United Kingdom) and incubated at 30 °C for 4–5 days. The top of the fungal colonies was covered with 1 mL of saline solution, and the colonies were gently scraped using a sterile loop to mix with the fluid. This suspension was collected in a sterile tube, and after waiting for 5–10 min at room temperature for the heavy particles to sink, the supernatant fluid was vortexed for 15 s. The conidia suspension was diluted with RPMI 1640 at a ratio of 1:50. Conidia were counted using a hemocytometer, and the amount of final inoculum was adjusted to yield 1–3 × 10^3 conidia/mL.

2.4. Antifungals

Seven antifungal drugs, namely MCZ, FLZ, VOR, KCZ, ITR, AMP B (Sigma-Aldrich, Madrid, Spain), and TER (Novartis Research Institute, Vienna, Austria) were procured from the manufacturers. Apart from FLZ, all drugs were dissolved in 10% DMSO (Sigma-Aldrich, Madrid, Spain). FLZ was dissolved in sterile distilled water. All drugs were diluted with RPMI 1640 growth media (Sigma-Aldrich, Madrid, Spain). Final concentrations were adjusted to 0.125–64 µg/mL for FLZ and 0.06–32 µg/mL for the remaining antifungal agents. Twofold serial dilutions of the antifungal drugs were dispersed to wells in 100-µL volumes. These dilutions were kept at −80 °C until use.

A broth microdilution test for the dermatophytes was performed in accordance with the CLSI M38-A2 document. Dilutions of the drug and the prepared inoculum were dispersed in wells of a microplate that contained 96 round-bottomed wells. In each microplate, inoculum suspension was added to the first 10 wells containing antifungal drugs. Each microplate had wells reserved for sterility control, which contained only the growth medium, and wells for growth control, which contained only the inoculum. The microplate was incubated at 35 °C for 4–5 days. The minimum inhibitory concentration (MIC) values were assessed visually. In comparison to the growth control well, the minimum drug concentration that caused 80% inhibition of growth was accepted as the MIC for TER, MCZ, KCZ, FLZ, ITR, and VOR. For AMP B, the minimum drug concentration that caused 100% growth inhibition was accepted as the MIC. For quality control, *Candida parapsilosis* 22019 standard strain was used.

2.5. Statistical analysis methods

Differences in MIC between antifungal agents and between organisms were evaluated using the Kruskal–Wallis test and Mann–Whitney U test with Bonferroni correction for multiple testing.

3. Results

The antifungal sensitivity of 66 strains belonging to the species *T. rubrum*, *T. interdigitale*, and *M. canis* are summarized in Table 1, including the MIC ranges and MIC50, MIC90, and GM values. Due to the inadequate number of *T. interdigitale* strains (n = 2), MIC90 and MIC50 values were not calculated for *T. interdigitale*.

TER and VOR produced the lowest MICs for all three species. ITR, KCZ, and MCZ also exhibited similar performance. Only AMP B exhibited high MIC. There was a significant difference regarding MIC90 values of antifungal agents between *M. canis* and *T. rubrum* ($P < 0.05$).

Table 1. In vitro activities of seven antifungal drugs against dermatophytes.

Species (number of strains tested)		Concentration (μg/mL)						
		AMP B	FLZ	ITR	KCZ	MCZ	TER	VOR
Microsporum canis (n = 9)	GM	1.26	0.18	0.09	0.10	0.07	0.06	0.06
	MIC50	2	0.125	0.125	0.125	0.06	0.06	0.06
	MIC90	2	0.5	0.125	0.125	0.06	0.06	0.06
	Range	(0.25–2)	(0.06–0.5)	(0.06–0.125)	(0.06–0.25)	(0.06–0.125)	(0.06–0.06)	(0.06–0.06)
Trichophyton interdigitale (n = 2)	GM	2.83	0.25	0.13	0.13	0.06	0.06	0.06
	Range	(2–4)	(0.06–1)	(0.06–0.25)	(0.06–0.25)	(0.06–0.06)	(0.06–0.06)	(0.06–0.06)
Trichophyton rubrum (n = 55)	GM	2.27	0.48	0.20	0.10	0.07	0.06	0.06
	MIC50	2	0.50	0.25	0.06	0.06	0.06	0.06
	MIC90	4	2	0.5	0.5	0.125	0.06	0.06
	Range	(0.25–8)	(0.06–4)	(0.06–1)	(0.06–2)	(0.06–0.25)	(0.06–0.06)	(0.06–0.06)
All organisms (n = 66)	GM	2.11	0.41	0.18	0.10	0.07	0.06	0.06
	MIC50	2.00	0.50	0.13	0.06	0.06	0.06	0.06
	MIC90	4.00	2.00	0.50	0.25	0.13	0,06	0,06
	Range	(0.25–8)	(0.06–4)	(0.06–1)	(0.06–2)	(0.06–0.25)	(0.06–0.06)	(0.06–0.06)

4. Discussion

Recently, a number of studies have examined in vitro detection of antifungal sensitivity in dermatophytes. However, previous authors had used the M38-A (9) and M38-P (10) protocols prior to publication of revised guidelines that specifically targeted molds. Both of these protocols target filamentous molds and required adaptation for dermatophytes. CLS M38-A2 has standardized antifungal sensitivity tests for dermatophytes by specifying factors like temperature and incubation time.

TER is an antifungal that can be used topically or systemically, and it was found to be the most potent drug in our study and in many other studies conducted in accordance with three different protocols. The results of our study are compared to numerous studies, which were conducted according to M38-A and M38-P documents, in Tables 2–4 (11–23). While this result is consistent with reports by Torres et al. (14) and Favre et al. (20) higher GM MIC values of VOR have been reported by Badali et al. (12), Silva et al. (17), and Adimi et al. (21). Some studies have reported higher GM MIC values for TER (23). For VOR, we found a GM MIC value of 0.06. In our study, the GM MIC range of ITR against *T. rubrum* isolates was 0.20 μg/mL. This result is consistent with other studies (13,17,19,20). Ansari et al., Badali et al., Gupta et al., and Torres et al. (11,12,14,23), however, reported higher GM MIC values of ITR.

In our study, ITR was more effective against *T. rubrum* isolates than KCZ, VOR, FLZ, MCZ, TER, and AMP B, with a MIC range of 0.06 μg/mL ($P < 0.002$). Against *M. canis*, inhibition by ITR was not significantly different compared to MCZ, TER, and VOR ($P > 0.489$). In *T. rubrum* there

Table 2. GM MIC values of ITR, KCZ, MCZ, VOR, TER, FLZ, and AMP B are compared to the results of previous studies that followed the CLSI M38-A2 protocol.

GM MIC value for *T. rubrum* (μg/mL)	Present study	Badali	Yenişehirli	Baghi et al.	Ansari	Silva
AMP B	2.27	2.82	0.07			
FLZ	0.48	45.25		20.8	27.47	11.20
ITR	0.20	0.6	0.20	0.18	0.06	0.22
KCZ	0.10		0.23			0.46
MCZ	0.07		0.16	3.31		
VOR	0.06	0.25				0.18
TER	0.06	0.04	0.06	0.07	0.017	0.06

Table 3. GM MIC values of ITR, KCZ, MCZ, VOR, TER, FLZ, and AMP B are compared to the results of previous studies that followed the CLSI M38-A protocol.

GM MIC value for *T. rubrum* (μg/mL)	Present study	Araujo et al.	Singh et al.	Afshari et al.	Adimi et al.
AMP B	2.27				
FLZ	0.48	7.6	1.92	23.77	11.05
ITR	0.20	0.10	0.59	0.135	0.06
KCZ	0.10	0.13		0.165	0.67
MCZ	0.07				
VOR	0.06				0.19
TER	0.06	0.11	0.006	1.097	0.172

Table 4. GM MIC values of ITR, KCZ, MCZ, VOR, TER, FLZ, and AMP B are compared to the results of previous studies that followed the CLSI M38-P protocol.

GM MIC value for *T. rubrum* (μg/mL)	Present study	Fermandez-Torres et al.	Favre et al.	Gupta et al.	Karaca et al.
AMP B	2.27	0.37			
FLZ	0.48	2.80	6.3	5.36	8
ITR	0.20	0.09	0.23	0.07	0.1
KCZ	0.10	0.14	0.22		0.5
MCZ	0.07	0.09	0.25		0.8
VOR	0.06	0.06	0.033		
TER	0.06	0.01	0.006	0.02	0.02

was no difference in inhibitory activity between KCZ and MCZ. AMP B exhibited the least effective antifungal activity against *M. canis* and *T. rubrum* ($P < 0.002$). The MIC values of FLZ against *T. rubrum* were significantly higher compared to those of KCZ, VOR, ITR, MCZ, and TER ($P < 0.002$). There was no significant difference between ITR and KCZ against *M. canis*.

While the GM MIC value of FLZ was 0.48 μg/mL, other studies have reported higher GM MIC values of FLZ (12,13,16,18,21,23). In our study, the GM MIC value was 0.10 μg/mL for KCZ and 0.07 μg/mL for MCZ. Our MIC result for KCZ is consistent with the results reported by Torres et al. (14), Afshari et al. (22), and Arajuo et al. (23) and our result for MCZ is consistent with the results reported by Karaca et al. (16). We found that the GM MIC value of amphotericin B was 2.27 μg/mL. This value was reported as 2.82 mg/mL by Badali et al. (12), whereas Torres et al. (14) found a much lower GM MIC value of AMP B of 0.37 μg/mL. According to our results, VOR, TER, ITR, MCZ, and KCZ showed notably low MIC values, and VOR and TER were the most potent among them.

Dermatophytoses are contagious and tend to become chronic. Currently sufferers constitute a significant group of patients in dermatology clinics. Accurate and rapid detection of the agent responsible for mycosis ensures effective treatment, especially in immunocompromised patients, and has other advantages like lower incidence of side effects and reduction in health care costs. Despite encouraging in vitro results, prevention of relapsing infections and improvements in clinical care will require further investigation of the reasons for relapses and treatment failures and effective monitoring of resistance development.

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