Eurasian J Med 2014; 46: 26-31 The Eurasian Journal of Medicine **Original Article**

Investigation of *In Vitro* Activity of Five Antifungal Drugs against Dermatophytes Species Isolated from Clinical Samples Using the E-Test Method

Beş Antifungal İlacın Klinik Örneklerden İzole Edilen Dermatofit Türlerine Karşı İn Vitro Aktivitesinin E-Test Yöntemi ile Araştırılması

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Abstract

Objective: Dermatomycosis is an infection with fungi related to the skin: glabrous skin, hair and/or nails. Oral treatment of fungal infections in dermatology has become a preferred modality for the management of these very common conditions. Although there are increasing numbers of antifungals available for treatment of dermatophytes, some cases and relapses have been unresponsive to treatment. The determination of fungus *in-vitro* antifungal susceptibility has been reported to be important for the ability to eradicate dermatophytes. It is necessary to perform antifungal susceptibility testing of dermatophytes. E-test (AB Biodisk, Sweden) is a rapid, easy-to-perform *in-vitro* antifungal susceptibility test. The aim of this study was to investigate the susceptibility of the different species of dermatophyte strains isolated clinical specimens to five antifungal agents using the E-test method.

Materials and Methods: A total of 66 specimens were collected from the nails, feet, inguinal region, trunk and hands. These strains tested MIC endpoints of E-test for amphotericin B, fluconazole, itraconazole, caspofungin, and ketoconazole were read after 72, and 96 hours incubation for each strain on RPMI 1640 agar.

Results: The dermatophytes tested included *Trichophyton rubrum* 43 (65.1%), *Trichophyton mentagrophytes* 7 (10.7%), *Microsporum canis* 5 (7.6%), *Trichophyton tonsurans* 5 (7.6%), *Epidermophyton floccosum* 4 (6.0%) and *Trichophyton violaceum* 2 (3.0%). The most active agent against all dermatophytes species was caspofungin with a minimal inhibitory concentration (MIC) range (μg/mL^{-1}) (0.02-3, 0.032-4, 0.125-0.50, 0.032-2, 0.25-0.50, 0.125-0.50) and it raconazole with an MIC range (μg/mL^{-1}) (0.038-1.5, 0.094-1.5, 1-32, 0.016-0.50, 0.25-0.50, 0.125-0.50). The least active agent was fluconazole with an MIC range (μg/mL^{-1}) (0, 19-48, 2-256, 2-8, 256, 256, 8-24).

Conclusion: E-test seems to be an alternative method to MIC-determination of antifungal drugs for dermatophytes species, since it is a less-laborious methodology and results could be obtained faster.

Key Words: Dermatophytes, E-test, antifungal

Özet

Amaç: Dermatomikozlar mantarlar tarafından oluşturulan deri, saç ve tırnakları tutan enfeksiyonlardır. Dermatolojide mantar enfeksiyonlarının oral tedavisi yaygın olarak tercih edilen yöntem haline gelmiştir. Dermatofitlerin tedavisi için kullanılan antifungallerin sayısının artmasına rağmen bazı durumlarda tedaviye yanıtsızlık ve nüksler gelişmektedir. *İn-vitro* antifungal duyarlılığın belirlenmesinin dermatofitlerin etkili olarak tedavi edilmesi için önemli olduğu bildirilmektedir. Antifungal duyarlılığın belirlenmesinde yöntemler önemlidir. E-test (AB Biodisk, İsveç) hızlı ve kolay uygulanabilir bir duyarlılık yöntemidir. Bu çalışmanın amacı, klinik örneklerden izole edilen farklı dermatofit türlerinin beş antifungal ilaca karşı duyarlılıklarını E-test yöntemi ile belirlemektir.

Gereç ve Yöntem: Tırnak, ayak, inguinal bölge, gövde ve el/bilek olmak üzere değişik anatomik bölgelerden alınan toplam 66 klinik örnek çalışmaya dâhil edildi. Bu örneklerden izole edilen dermatofit suşlarının duyarlılıkları, amfoterisin B, flukonazol, itrakonazol, kaspofungin ve ketokonazol'e karşı RPMI 1640 agar besiyerinde 72 ve 96 saat süre ile E-test yöntemi kullanılarak çalışıldı.

Bulgular: Çalışmaya dâhil edilen dermatofit türleri *Trichophyton rubrum* 43 (%65,1), *Trichophyton mentagrophytes* 7 (%10,7), *Microsporum canis* 5 (%7,6), *Trichophyton tonsurans* 5 (%7,6), *Epidermophyton floccosum* 4 (%6,0) and *Trichophyton violaceum* 2 (%3,0) olarak belirlendi. Tüm dermatofit türlerine karşı en etkili antifungaller (0,02-3, 0,032-4, 0,125-0,50, 0,032-2, 0,25-0,50, 0,125-0,50) MIC (μg/mL^{-1}) aralıkları ile kaspofungin ve (0,038-1,5, 0,094-1,5, 1-32, 0,016-0,50, 0,25-0,50, 0,125-0,50) MIC (μg/mL^{-1}) aralıkları ile itrakonazol olarak bulundu. En az etkili antifungal ise (0, 19-48, 2-256, 2-8, 256, 256, 8-24) MIC (μg/mL^{-1}) aralıkları ile flukonazol olarak bulundu.

Sonuç: E-test dermatofit türlerinin antifungal duyarlılığının belirlenmesinde daha az zahmetli ve sonuçların daha hızlı elde edilebilir olması ile alternatif bir yöntem gibi görünmektedir.

Anahtar Kelimeler: Dermatofitler, E-test, antifungal

Received: May 21, 2013 / **Accepted:** August 2, 2013

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doi:10.5152/eajm.2014.05

Introduction

Dermatophytes are a specialized group of fungi, which effect keratinous tissue of humans and other vertebrates, causing superficial infections. The organisms belong to three genera, Trichophyton, Epidermophyton, and Microsporum. Infections caused by these fungi are among the most prevalent cutaneous infections globally and the recent increase in the number of patients with immunocompromised states, such as AIDS, diabetes mellitus, cancer and organ transplantation has given these infections more prominence [1-6].

The treatment of dermatophytosis is based on the use of topical and systemic antifungal agents. In recent years, a number of safe and highly effective antifungal agents have been introduced into clinical practice. Although an increasing number of antimycotics have become available for the treatment of dermatophytosis, there are reports suggesting recalcitrant to therapy or possibly resistance of dermatophytes to antimicrobial agents. In order to predict the ability of a given antimycotic agent to eradicate dermatophytes and help managing patients, determination of the in vitro antifungal susceptibility of dermatophytes would be helpful in understanding a failed or successful treatment. However, not all species have the same susceptibility pattern and it may be necessary to perform in vitro susceptibility testing for selection and monitoring of antifungal therapy. Although a reference method is not yet available, various techniques have been used to test dermatophytes, including broth macro- and micro dilution methods, agar dilution and disc diffusion. However, these methods are time-consuming and labour-intensive, and are not practical for the clinical laboratory. Therefore, simple alternative approaches are needed [1, 3, 4, 6-8].

The E-test is a simple, agar-based, quantitative minimal inhibitory concentration (MIC) method. The reagent consists of a thin, calibrated plastic strip with a predefined, exponential and continuous gradient of antifungal agent across 15 two-fold dilutions. The E-test has been satisfactorily used to test bacteria, yeasts and moulds. However, there is limited data available on the performance of the E-test for antifungal susceptibility of dermatophytes [4, 9-11].

The aim of this study was to investigate the susceptibility of the different species of dermatophyte strains isolated clinical specimens to five antifungal agents (amphotericin B, fluconazole, itraconazole, caspofungin, and ketoconazole) using the E-test method.

Materials and Methods

Strains and Specimens: Sixty-six strains were isolated from infected skin and nails in the Microbiology and Clinical Microbiology Department of School of Medicine, Ataturk University. Isolates were collected over a one-year period in Mycology Laboratory. They included *T. rubrum*, *T. mentagrophytes*, *M. canis*, *T. tonsurans*, *E. floccosum* and *T. violaceum*. All strains were identified by standard methods, which included identification based on the macroscopic and microscopic characteristics of the culture strains. Additional tests included those for the ability to produce a red pigment when the strains were grown on Potato Dextrose Agar (PDA) and for the ability to produce urease, as well as the hair perforation test. Strains were stored -70^{0}C until the time of use, and prior to testing were sub-cultured on PDA at 28^{0}C for 15 days to ensure optimal growth characteristics [1, 3, 6]. All procedures in the experimental protocol were approved by The Ethics Committee of Medical Faculty.

E-Test Method

Medium: The test was performed in RPMI 1640 medium with L-glutamine, although without bicarbonate (Gibco, New York, USA), pH 7.0 supplemented with 2% glucose, buffered 0.165 M morpholinepropanesulfonic acid (MOPS) (Fisher Biotech, New Jersey, USA) and 1.8% agar (Difco, Sparks, USA). The 15-cm diameter petri plates contained RPMI 1640 at a depth of 4.0 mm [4].

Antifungal Agents: E-test strips were obtained from AB Biodisk (Solna, Sweden) and stored at -20^{0}C until tests were performed. The concentrations assayed ranged from 0.002 to 32.000 μg/mL^{-1} for amphotericin B, itraconazole, caspofungin, and ketoconazole and 0.016 to 256.000 μg/mL^{-1} for fluconazole.

Procedure: All isolates were tested against five antifungal agents using the E-test according to the manufacturer's instructions. The inoculums suspensions were prepared and adjusted to 65-70% transmittance at a wavelength of 530 nm corresponding to a concentration of 10^5-10^6 cfu/mL^{-1} verified by quantitative plate counts. The RPMI agar surface was inoculated by dipping a sterile swab into the inoculums suspension and streaking it evenly in three directions. After excess moisture was absorbed into the agar and the surface was completely dry, an E-test strip was applied to each plate. The plates were incubated at 28^{0}C and the results were read at 72-96 hour [4].

Determination of MIC endpoints: In general, MIC was defined as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the MIC scale on the E-test strip. When a double halo of growth was observed, the MIC was read at the point where growth was completely inhibited. When different intersections were observed on either side of the strip, the highest MIC value was read [4].

Results

The isolated dermatophytes were obtained from the toenails 16 (24.2%), feet 33 (50.0%), inguinal region 7 (10.7%), trunk 5 (7.6%) and hands 5 (7.6%). The distribution of isolated species 66 dermatophytes were *T. rubrum* 43 (65.1%), *T. mentagrophytes* 7 (10.7%), *M. canis* 5 (7.6%), *T. tonsurans* 5 (7.6%), *E. floccosum* 4 (6.0%) and *T. violaceum* 2 (3.0%) (Table 1).

All strains tested grew well on RPMI glucose, supplement agar plated. They were read in the E-test method after 96 hours of incubation, except in the case of *T. mentagrophytes*, which required only 72 hours of incubation.

Table 2 summarizes the in vitro susceptibilities of 66 clinical isolates of dermatophytes to five antifungal agents as determined by E-test. The most active agent against all dermatophytes species was caspofungin with an MIC range (μg/mL^{-1}) (0.02-3, 0.032-4, 0.032-4, 0.125-0.50, 0.25-0.50, 0.125-0.50) and itraconazole with an MIC range (μg/mL^{-1}) (0.038-1.5, 0.094-1.5, 1-32, 0.016-0.50, 0.25-0.50, 0.125-0.50). The least active agent was fluconazole with an MIC range (μg/mL^{-1}) (0,19-48, 2-256, 2-8, 256, 256, 8-24).Test results of the susceptibility to amphotericin B and ketoconazole were as follows; respectively, 0,012-8, 0,19-8, 0,50-3, 0,125-6, 32, 0,75 and 0,032-8, 0,064-8, 32, 32, 32, 32.

In general, the species of dermatophytes showed similar patterns of susceptibility to each antifungal agent tested. High MIC values were found for some isolates, two dermatophytes strains (1 *T. rubrum and 1 T. mentagrophytes)* had MICs of caspofungine of 32 μg/mL, 16 strains (11 *T. rubrum*, 4 *E. floccosum* and 1 *T. mentagrophytes*) had MICs of Amphotericin B of 32 μg/mL, 53 strains (36 *T. rubrum*, 5 *T. tonsurans*, 4 *E. floccosum*, 2 *M. canis, and 6 T. mentagrophytes*) had MICs of fluconazole of 256 μg/mL, 2 strains (2 *M. canis)* had MICs of itraconazole of 32 μg/mL, and 33 strains (18 *T. rubrum*, 1 *T. tonsurans,* 4 *E. floccosum,* 5 *M. canis,* 2 *T. violaceum*, and 3 *T. mentagrophytes*) had MICs of ketoconazole of 32 μg/mL. Table 2 summarizes the MIC ranges, concentrations inhibiting 50% (MIC 50) and 90% (MIC 90) of the isolates of the five antifungal drugs against 66 strains of dermatophytes.

Discussion

Infections caused by dermatophytes occur worldwide and can be very severe and difficult to treat in patients whose immunological response is impaired. These infections represented an important public health problem as yet unresolved [4, 7].

Dermatophytes are responsible for the majority of fungal infections involving the skin, hair and nails. They comprise a phylogenetically closely related group of genera with numerous species. They attack the keratinized tissues and cause a wide spectrum of clinical manifestations that vary from mild to severe [6].

The distribution of the dermatophytes and their etiological agents has unequal frequencies, with variations of their prevalence according to the countries and even the regions of the same country. In this study, *T. rubrum* was the most frequently isolated organism 43 (65.1%), followed by *T. mentagrophytes* 7 (10.7%), *M. canis* 5 (7.6%), *T. tonsurans* 5 (7.6%), *E. floccosum* 4 (6.0%) and *T. violaceum* 2 (3.0%). These results are in agreement with many other local [3, 12-19] and international studies [1, 4, 7, 9, 20-25].

Most superficial infections caused by dermatophytes can be rapidly eradicated with topical and systemic antifungals. Oral antifungal therapy with newer agents, such as terbinafine, itraconazole and fluconazole, is the treatment of choice for dermatophytosis that does not respond to topical therapies. The activity spectrum to these drugs is variable, leading to treatment failure in 25-40% of treated patients, possibly due to poor patient compliance, lack of drug penetration into nail, medication bioavailability or drug interactions and resistance [26].

In vitro analysis of the antifungal activity of anti-fungal agents enables comparison between different antimycotics, which in turn may clarify the reasons for lack of clinical

Table 1. Isolated dermatophyte strains in relation to localization

			Localization				
Dermatophytes	n	%	Toe nail	Foot	Inguinal region	Trunk	Hands
T. rubrum	43	65.1%	12	24	4	3	-
T. mentagrophytes	7	10.7%	1	2	-	2	2
M. canis	5	7.6%	1	3	-	-	1
T. tonsurans	5	7.6%		2	3	-	-
E. floccosum	4	6.0%	2	2	-	-	-
T. violaceum	2	3.0%	-	-	-	-	2
Total	66	100.0%	16 (24.2%)	33 (50.0%)	7 (10.7%)	5 (7.6%)	5 (7.6%)

Table 2. Susceptibility data for dermatophytes species against five antifungal agents using the E-test method

Species (n)	Antifungal agent	MIC range*	MIC_{50}	MIC_{90}
T. rubrum (43)	Amphotericin B	0.012-8	0.50	1.5
	Fluconazole	0.19-48	-	-
	Itraconazole	0.038-1.5	0.50	0.19
	Caspofungine	0.02-3	1	0.064
	Ketoconazole	0.032-8	-	-
T. mentagrophytes (7)	Amphotericin B	0.19-8	0.70	4
	Fluconazole	2-256	-	-
	Itraconazole	0.094-1.5	0.25	1.5
	Caspofungine	0.032-4	0.25	2
	Ketoconazole	0.064-8	2	8
M. canis (5)	Amphotericin B	0.50-3	0.50	1
	Fluconazole	2-8	-	-
	Itraconazole	1-32	-	-
	Caspofungine	0.125-0.50	0.50	0.125
	Ketoconazole	32	-	-
T. tonsurans (5)	Amphotericin B	0.125-6	0.50	0.50
	Fluconazole	256	-	-
	Itraconazole	0.016-0.50	0.125	0.125
	Caspofungine	0.032-2	0.032	0.032
	Ketoconazole	32	-	-
E. floccosum (4)	Amphotericin B	32	-	-
	Fluconazole	256	-	-
	Itraconazole	0.25-0.50	0.25	0.50
	Caspofungine	0.25-0.50	0.25	-
	Ketoconazole	32	-	-
T. violaceum (2)	Amphotericin B	0.75	-	-
	Fluconazole	8-24	-	-
	Itraconazole	0.125-0.50	-	-
	Caspofungine	0.125-0.50	-	-
	Ketoconazole	32	-	-

MIC: Minimal inhibitory concentration
*MIC ($\mu g/mL^{-1}$)

response and assist clinicians in choosing an effective therapy for their patients. However, it is important that the methodologies used for in vitro testing be standardized to facilitate the establishment of quality control parameters and interpretive break points [27].

Currently, no reference method has been established to test drug susceptibilities of dermatophytes. The development of simple and reproducible techniques is required for clinical testing of these important pathogens. The E-test is a new and promising method with broad applications in clinical laboratory practice, and is supported by the results of extensive testing of bacteria and yeasts. However, there are only a few reports describing the use of this method for dermatophytes [4, 9, 20-22].

In this study, we investigated MIC values of five antifungal agents (amphotericin B, fluconazole, itraconazole,

caspofungin, and ketoconazole) to the different species of dermatophyte strains isolated clinical specimens using the E-test method.

In our study, the most active agent against all dermatophytes species was caspofungin with an MIC range (μg/mL^{-1}) (0.02-3, 0.032-4, 0.125-0.50, 0.032-2, 0.25-0.50, 0.125-0.50) and itraconazole with an MIC range (μg/mL^{-1}) (0.038-1.5, 0.094-1.5, 1-32, 0.016-0.50, 0.25-0.50, 0.125-0.50). The least active agent was fluconazole with an MIC range (μg/mL^{-1}) (0.19-48, 2-256, 2-8, 256, 256, 8-24). Test results of the susceptibility to amphotericin B and ketoconazole were as follows; respectively, 0.012-8, 0.19-8, 0.50-3, 0.125-6, 32, 0.75 and 0,032-8, 0,064-8, 32, 32, 32, 32.

With respect to itraconazole, all of *T. rubrum* isolates were inhibited in concentrations ranging from 0.038 to 1.5 μg/mL^{-1}. The other species, except *M. canis* showed similar sensitivity ranges. Two *M. canis* strains had MICs of itraconazole of 32 μg/mL. However, for fluconazole, we observed that high MIC values. Fifty-three strains (36 *T. rubrum*, 5 *T. tonsurans*, 4 *E. floccosum*, 2 *M. canis, and 6 T. mentagrophytes*) had MICs of fluconazole of 256 μg/mL. In general, our data are in agreement with studies of Don Santos et al. [22], Fernandez-Torres et al. [4], Silva-Barros et al. [9], Kang et al. [21] and Abdel-Aal et al. [20].

Caspofungin the other most active agents for all dermatophytes species in our study with an MIC range (0.02-3 for *T. rubrum*, 0.032-4 for *T. mentagrophytes*, 0.032-4 for *M. canis*, 0.032-2 for *T. tonsurans*, 0.25-0.50 for *E. floccosum*, 0.125-0.50 for *T. violaceum*).

In our study, 33 (50%) isolates of tested dermatophytes by E-test (18 *T. rubrum*, 1 *T. tonsurans,* 4 *E. floccosum,* 5 *M. canis,* 2 T. *violaceum*, and 3 *T. mentagrophytes*) were resistant with an MIC range 32 μg/mL of ketoconazole. These results were obtained other researchers [4, 9, 20-22].

Amphotericin B, the other drug with an MIC range (0.012-8, 0.19-8, 0.50-3, 0.125-6, 32, 0.75) in the present study. 16 strains (11 *T. rubrum*, 4 *E. floccosum* and 1 *T. mentagrophytes*) had MICs of amphotericin B of 32 μg/mL. Kang et al.[21] observed that amphotericin B was 0.094~0.5 μg/mL on *T. rubrum*, 0.032~1.0 μg/mL on *T. mentagrophytes*, 0.19 μg/mL on *M. canis*, and 0.032 μg/mL on *M. gypseum*.

Antifungal susceptibility testing is a dynamic field of medical mycology. Development and standardization of antifungal susceptibility test have shown remarkable progress in the field of medical mycology [6], although, studies using the E-test method for dermatophytes susceptibilities is not yet sufficient. In a limited number of studies, showed that E-test seems to be an alternative method to MIC-determination of antifungal drugs for dermatophytes, since it is a less-laborious methodology and results could be obtained faster [4, 9, 21, 22].

In conclusion, this study showed that the E-test represented a simple and efficacious method for antifungal susceptibility testing of dermatophytes. Regarding its performance, the E-test was not labour demanding, was easy to interpret, and with the potential of being used as an alternative assay for azole antifungal susceptibility testing of dermatophytes.

Conflict of Interest: No conflict of interest was declared by the authors.

Peer-review: Externally peer-reviewed.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Author Contributions: Concept - A.E.A., N.Y.; Design - A.E.A., N.Y.; Supervision - A.E.A., A.A.; Funding - A.E.A., ; Materials - A.A., A.E.A.; Data Collection and/ or Processing - S.G.G., A.E.A., A.A., N.Y.; Analysis and/or Interpretation - A.E.A., N.Y.; Literature Review - A.E.A., N.Y.; Writer - A.E.A., N.Y.; Critical Review - A.E.A., A.A., N.Y.

Financial Disclosure: The authors declared that this study has received no financial support.

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