Original article

In vitro activity of six antifungal drugs against clinically important dermatophytes

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Abstract

Introduction and objective: Dermatophytosis is a common fungal disease which involves the keratinized tissue. Several antifungal agents can be used to manage these infections. Unfortunately, drug resistant can result in treatment failure. The disk diffusion *in vitro* assay is a simple method that can be used to evaluate antifungal susceptibility in dermatophytes. The main aim of this study was to evaluate the antifungal activity of six antifungal drugs against several fresh clinical dermatophyte Iranian isolates.

Materials and methods: Forty clinical dermatophytes were isolated from patients suspected of having active dermatophytosis. Paper disks containing terbinafine, griseofulvin, clotrimazole, miconazole, fluconazole and ketoconazole were used in the disk diffusion method to evaluate the *in vitro* activity of the antifungal agents by measuring the mean diameter of inhibition around the disks.

Results: The isolates belong to three genera and eight species as: *Trichophyton mentagrophytes* 13(32.5%), *T. rubrum* 8(20%), *Epidermophyton floccosum* 7(17.5%), *T. violaceum* 4(10%), *Microsporum gypseum* 3(7.5%), *T. tonsurans* 2(5%), *T. verrucosum* 2(5%), *T. schoenleinii* 1(2.5%), and an unknown dermatophyte 1(2.5%). No isolates were resistant to clotrimazole and miconazole.

Conclusion: This study revealed that clotrimazole, miconazole, terbinafine, and griseofulvin were the most ideal antifungal drugs for the treatment of dermatophytosis. Disk diffusion method is a simple and valuable method for the evaluation of antifungal susceptibility of dermatophytes.

Keywords: Dermatophytosis, Disk diffusion, Antifungal agents, Dermatophyte

Introduction

Dermatophytes are responsible for the majority of fungal infections involving skin, hair and nails. They comprise a phylogenetically closely related group of genera with numerous species [1]. They attack the keratinized tissues and cause a wide spectrum of clinical manifestations that vary from mild to severe. Dermatophytosis is one of the most

common fungal infections worldwide. In Iran, dermatophytosis is common and has been reported from different parts of the country [2].

There are several antifungal drugs used to treat dermatophytosis. Some infections respond well to topical antifungal therapy, whereas others like tinea capitis, tinea unguium (nail infection), and more extensive or severe types may require systemic therapy [3]. Occasionally, in some cases, antifungal therapy is a failure because of resistance to the antifungal drugs by the fungi. Therefore, we believe, it is essential to evaluate the resistant dermatophytes using a standardize, simple and reproducible *in vitro* assay to determine the antifungal activity of drugs against isolates [4].

In vitro antifungal susceptibility tests are now mainly used for epidemiological surveys, determination of the degree of antifungal activity, and the prediction of clinical outcome based upon an optimization of antifungal therapy [5]. Several methods have been developed for testing antifungal agents against this group of pathogens [5,6]. Multicenter studies to develop a standardized antifungal susceptibility assay were initiated by the Clinical and Laboratory Standards Institute (CLSI, formerly 'National Committee for Clinical Laboratory Standards', NCCLS) in 1983. Dilution tests are widely used in macro- and micro-assays, but these methods are difficult to be used in most laboratories. Recently, studies were done to establish a simple method to solve this problem [5,7,8].

The agar-based disk diffusion (DD) susceptibility method for dermatophytes is simple, inexpensive, and does not require specialized equipment [9]. The disk diffusion method has a good correlation with the reference dilution assay [3,10,11]. The main aim of this study was to determine *in vitro* activity of six antifungal drugs that are most commonly used to treat dermatophytosis; Griseofulvin (GF), Miconazole (MIZ), Terbinafine (TBF), Clotrimazole (CTZ), Fluconazole (FLZ) and Ketoconazole (KTZ).

Materials and methods

The isolates

The clinical dermatophyte isolates were isolated from patients suspected of having a dermatophytosis who were referred to the medical mycology laboratory, Shiraz Medical School in Shiraz, southern Iran. Samples were taken from infected areas and cultivated on Mycosel agar (Hi-media, India) that was incubated at 25°C for 2-3 weeks. Identification of the isolates was based on gross colony characteristics and microscopic morphology of their micro- and macroconidia and accessory structures. The isolates were then transferred to sterile distilled water (DW) in vials and stored at room temperature as stocks.

Preparation of the antifungal disks

All antifungal drugs were obtained as standard powder. The stocks were prepared by dissolving the powders in their specific solvents (DMSO, water, ethanol), after which, they were loaded into blank paper disks at the following potencies: clotrimazole (10µg/disk), terbinafine (30µg/disk), griseofulvin (25µg/disk), fluconazole (25µg/disk), ketoconazole (10µg/disk) and miconazole (10µg/disk) according to antifungal disks potency of Rosco Diagnostica Company (Neo-sensitabs, Denmark). Amphotericin B and clotrimazole disks (Hi-media, India), and standard strain of *T. rubrum* (gift from professor Zainie, Institute of Health and Research, Tehran) were used as controls.

Preparation of the inocula

The isolates were transferred from DW stocks to Mycosel agar and then sub-cultured to potato dextrose agar (Merck, Germany) to enhance sporulation. Seven

day-old cultures were covered with 1ml DW and the colonies were probed with the tip of a sterile Pasteur pipette to obtain a mixture of mycelium and conidia. The suspensions were transferred to sterile tubes and allowed to sediment for 30 minutes and then adjusted with a spectrophotometer set at 65% transmittance and 530nm [3].

Disk diffusion assay

All the tests were performed according to Esteban *et al*. [3]. The inoculum was evenly spread on the surface of 10cm Petri dishes containing Sabouraud dextrose agar medium (Merck, Germany) and exposed to air dry. Then, the antifungal disks were applied to the plates, after which the plates were incubated at 25°C for 5-10 days. After the colonies grew, the zones of inhibition around the disks were measured and recorded. Criteria of susceptibility and resistance of antifungal disks were measured according to following table 1. All tests were performed in duplicate and Microsoft SPSS was used for data analysis.

Table 1: Criteria of susceptibility and resistance of antifungal disks

Antifungal drugs	Potency	Zone diameter in mm		
		Sensitive	Intermediate	Resistance
Clotrimazole	10 µg	≥20	19-12	≤11
Fluconazole	25 µg	≥22	21-15	≤14
Griseofulvin	25 µg	≥10	-	No zone
Ketoconazole	15 µg	≥30	29-23	≤22
Miconazole	10 µg	≥20	19-12	≤11
Terbinafine	30 µg	≥20	19-12	≤11

Results

A total of forty species of dermatophytes were isolated and identified. The isolates belong to three genera and eight species as follows: *T. mentagrophytes* 13(32.5%), *T. rubrum* 8(20%), *E. floccosum* 7(17.5%), *T. violaceum* 4(10%), *M. gypseum* 3(7.5%), *T. verrucosum* 2(5%), *T. tonsurans* 2(5%), *T. schoenleinii* 1(2.5%) and *Trichophyton* species 1(2.5%).

Test results of the susceptibility to antifungal drugs were as follows: Ketoconazole: 31 (77.5%) susceptible, 4(10%) intermediate, 5 (12.5%) resistant. Griseofulvin: 3 (7.5%) resistant, 37 (92.5%) sensitive. Miconazole: 36 (90%) sensitive, 4 (10%) intermediate. Terbinafine: 1 (2.5%) resistant, 39 (97.5%) sensitive. Clotrimazole: 39 (97.5%) susceptible, 1 (2.5%) intermediate. Fluconazole: 39 (97.5%) resistant, 1 (2.5%) intermediate. Regarding the data, it was revealed that clotrimazole and terbinafine were the most effective antifungal drugs and fluconazole had the poorest activity (Fig. 1).

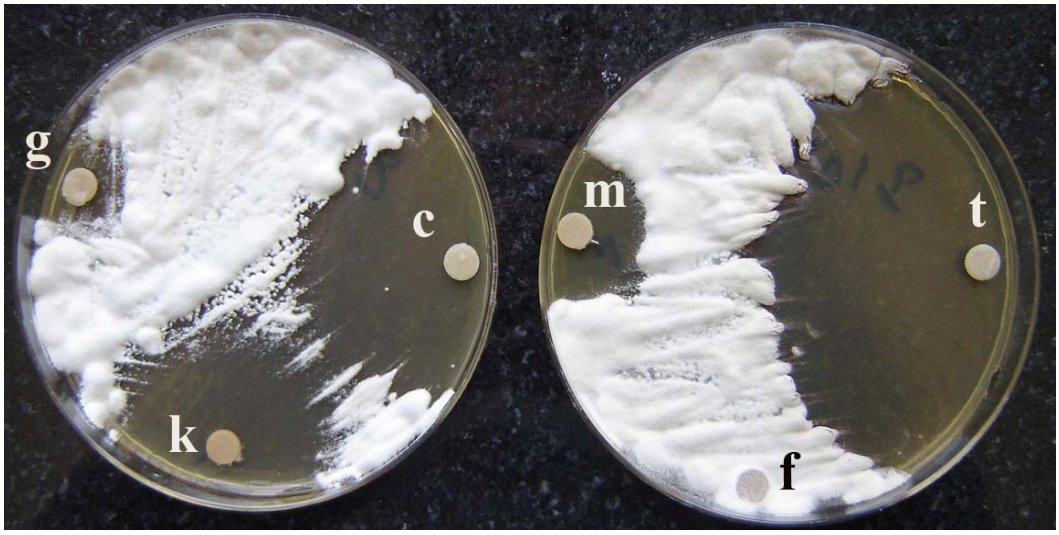

Fig. 1: Sensitivity *T. mentagrophytes* to tested antifungals drugs
g, Griseofulvin; k, Ketoconazole; c, Clotrimazole; m, Miconazole; f, Fluconazole; t, Terbinafine

Discussion

Antifungal susceptibility testing is a dynamic field of medical mycology [5]. Development and standardization of antifungal susceptibility tests have shown remarkable progress in the field of medical mycology. Despite the many guide lines that NCCLS have published for susceptibility tests of moulds (M-27A, M-28A), there is no clear method and routine test for the evaluation of dermatophyte antifungal activity [5,7,8]. The agar diffusion method is a practical, agar-based method which enables the determination of the activity of various antifungal drugs against various fungal genera and species. Broth macro- and micro-dilution assays can be used to determine antifungal susceptibility of dermatophytes, but these methods are expensive and require specific media and equipment such as RPMI, MOPS buffer, and micro plate trays.

The standard disk diffusion assay constitutes a good model to be used for investigational purposes to test other fungal genera and drugs as well. This assay can be adapted for routine diagnosis in the laboratory and for assessment of dermatophyte resistance against antifungal drugs. Some studies have focused on the comparison of the disk diffusion method with the reference micro-dilution method. These studies suggest that disk diffusion is a reproducible method which in general shows good correlation with the reference method for micro-dilution antifungal susceptibility test [12,13].

Other studies such as the one done by Singh *et al.* [9] could not find a significant correlation between micro-dilution and disk diffusion methods, probably due to their use of Dermasel agar medium. This medium is unacceptable for antifungal susceptibility testing. In our study, clotrimazole, terbinafine and ketoconazole had large inhibition zones around the disks; clotrimazole had the best activity against the isolates. Clotrimazole is one of the oldest antifungal drugs formulated as a topical for use against dermatophytosis.

Although clotrimazole is effective against most cases of dermatophytosis, it is not suitable for severe infections involving hair and nail, which need additional systemic therapy. Terbinafine was the second most antifungal drug. This drug has

two forms (topical and oral) and has many advantages [9,11]. We believe that this drug should be considered as the first choice for treatment of dermatophytosis, especially in nail and hair invasion when its side effects are tolerated. In this study, fluconazole, in spite of using drug potency up to 50 microgram per disk, had poor activity on isolates tested. In most isolates, no inhibition zones were observed around the disks.

There are many studies indicating that fluconazole had less activity against dermatophytes [9,11,14]. Our data is in agreement with those reports. This is perhaps because fluconazole is a triazole, and Sabouraud dextrose agar has components that can interfere with the test. Moreover, *in vitro* determination of antifungal activity of fluconazole against dermatophytes has variable results because of the use of different methods and media [9,11,13,15].

We see such variations even between broth macro- and micro-dilution methods in the Siqueira *et al*. [16] study. Their results lacked a good and significant correlation with each other. We recommend using the other susceptibility test methods with RPMI medium instead of disk diffusion for fluconazole. The other antifungal drugs used in this study included miconazole, griseofulvin and ketoconazole that showed good activity. Griseofulvin, of course, is only administrated orally and should be used with the other topical antifungal drugs to get the best result.

Conclusion

We have demonstrated that clotrimazole was the best antimycotic agent against dermatophytes followed by terbinafine and griseofulvin. The disk diffusion method is a simple, reliable, inexpensive and easily adaptable assay which is more practical, simple and easier test in comparison with M38-P and M38-A reference methods.

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