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In Vitro Antifungal Susceptibility Testing of 5 Antifungal Agents against Dermatophytic Species by CLSI (M38-A) Micro Dilution Method

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Abstract

Cases of dermatophytoses have increased over the past few decades. In the last few years, a number of newer less toxic antifungal drugs have become available for clinical use. The increased use of antifungal, often for prolonged periods, has led to the recognition of the phenomenon of acquired antifungal resistance among previously susceptible strains or species and to the increased incidence of infections with less common species. Our study mainly focused on the *in vitro* susceptibility of clinical isolates of dermatophytes against frequently used 5 antifungal agents. The microbroth dilution method was performed according to CLSI standards. In the present study antifungal susceptibility testing was done by micro dilution method of dermatophytes against 5 antifungal agents namely, ketoconazole (imidazoles) fluconazole, itraconazole (triazoles), griseofulvin and terbinafine and their activity against significant number of strains, representing a wide spectrum of dermatophyte species is assessed.

Dermatophytic strains: A total of 119 strains of dermatophytes belonging to 10 species were tested. They were *T. rubrum* (n=40), *T. mentagrophytes* (n=19*), T. violaceum* (n=15), *M. gypseum (*n=12), *E. flocossum* (n=9), *M. audouinii* (n=8), *T. schoenleinii* (n=5), *M. canis* (n=5), *T. tonsurans* (n=4) and *T. verrucosum* (n=2). The MIC ranges of all the 119 isolates of dermatophytes tested for antifungal susceptibility showed that terbinafine had the lowest MIC range of 0.001 to 0.64 µg/ml followed by ketoconazole at a MIC range of 0.01-3.84 µg/ml. The itraconazole showed a MIC range of 0.082-20.45 µg/ml whereas the griseofulvin and fluconazole showed a highest MIC range of 0.32-5.12 µg/ml. The MIC_{50} of Terbinafine was low at 0.02 µg/ml followed by Ketaconazole 0.24 µg/ml. The MIC_{50} of itraconazole and griseofulvin was1.28 µg/ml .The highest MIC_{50} with 2.56 µg/ml was recorded for Fluconazole. The MIC90 of terbinafine was low at 0.32 µg/ml followed by Ketaconazole with 1.92 µg/ml. The MIC_{90} Itraconazole was 2.50 µg/ml and for griseofulvin it was 2.56 µg/ml. The highest MIC_{90} of flucanozole was high at 10.24 µg/ml. In our study, we observed that terbinafine had the lowest MIC values compared to ketoconazole, itraconazole, griseofulvin and fluconazole. Hence the efficacy of terbinafine is higher followed by ketoconazole when compared with other drugs. This study helps in choosing a right antifungal drug for treating tinea infections.

Keywords: Antifungal; Dermatophytes; Itraconazole; Tineacapitis

Introduction

Dermatophytes are fungi that can cause infections of the skin, hair, and nails due to their ability to utilize keratin. The organisms colonize the keratin tissues [1] and inflammation is caused by host response to metabolic by-products. The dermatophytes are included in three fungal genera viz,:1. Epidermophyton: This genus consists of 2 species, one of which is a pathogen 2. Microsporum: There are 19 described species but only 9 are involved in human or animal infections. 3. Trichophyton: There are 22 species, most causing infections in humans or animals [2].

The infections caused by dermatophytes are known as ringworm or tinea infections. Tinea means "ringworm" or "moth-like". Dermatologists use the term to refer to a variety of lesions of the skin or scalp. Tinea corporis – ringworm of glabrous skin in which small lesions occurring anywhere on the body Figure 1 (Plate 5). Tinea pedis -"athlete's foot", ringworm infection of toe webs and soles of feeT. Tinea unguium (onychomycosis) - ringworm of nails Figure 1 (Plate-6). Tinea capitis – ringworm of head or scalp Figure 1 (Plate 1 and 2). Frequently found in children. Tinea cruris - "jock itch" ringworm of the groin, perineum or perianal area (Plate-3). Tinea facie Figure 1 (Plate 4) ringworm of the the face and tinea mannum Figure 1 (Plate 7) ringworm on hands [3].

Occasionally the organisms do invade the subcutaneous tissues, resulting in kerion development (Plate-1). The organisms are transmitted by either direct contact with infected host (human or animal) or by direct or indirect contact with infected exfoliated skin or hair in combs, hairbrushes, clothing, furniture, theatre seats, caps bed linens, towels, hotel rugs, and locker room floors. Depending on the species the organism may be viable in the environment for up to 15 months. There is an increased susceptibility to infection when there is a preexisting injury to the skin such as scars, burns, wounds and during marching, high temperature and humidity.

In the last two decades the incidence of infections caused by dermatophytes and other fungi has increased considerably [4]. With an increasing variety of drugs available for the treatment of dermatophytoses, the need for a reference method for the testing of the antifungal susceptibilities of dermatophytes has become apparent [5]. Establishment of a reference susceptibility testing method may allow the clinician to select the appropriate therapy for the treatment of infections caused by dermatophytic fungi. Our study mainly focused on the *in vitro* susceptibility of clinical isolates of dermatophytes. The microbroth dilution method was performed according to CLSI standards (previously the NCCLS method). In the present study antifungal susceptibility testing was done by micro

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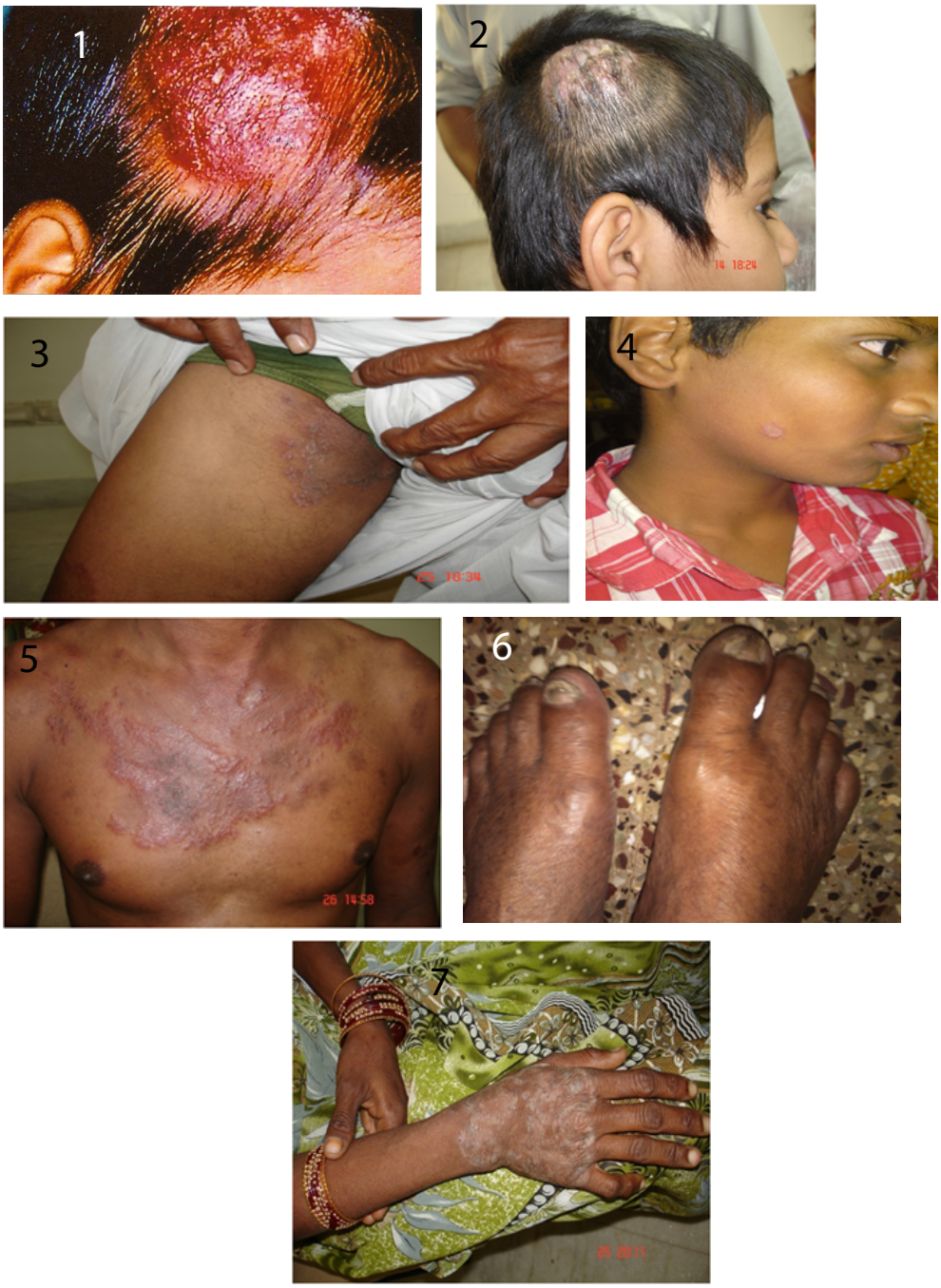

Figure 1: Clinical types in dermatophytosis.
Plate-1: Tinea capitis with kerion
Plate-2: Tinea capitis
Plate-3: Tinea cruris
Plate-4: Tinea facie
Plate-5: Tinea corporis
Plate-6: Tinea pedis (onychomycosis)
Plate-7: Tinea mannum

dilution method of dermatophytes against 5 antifungal agents namely, ketoconazole (imidazoles) fluconazole, itraconazole (triazoles), griseofulvin and terbinafine and their activity against significant number of strains, representing a wide spectrum of dermatophytic species is assessed.

Materials and Methods

Study group

The present study was conducted on 400 clinically diagnosed patients with dermatophytoses who visited as our patients at Ramesh

Skin Hospital (Dr. Ramesh, DermatologisT.) during the two-year period: January 2008 to December 2010. Most of the patients belong to low and middle socioeconomic groups coming from Warangal town and surrounding villages of Warangal districT. As the Warangal climate is mostly hot and humid the patients with dermatophytosis are more in number. The data from the patients was collected by supplying a data sheet regarding name, age, sex, address, occupation, family history, and socioeconomic background, duration of illness personal contact at home, work place/school and involvement of more than one site.

The samples from patients were collected in aseptic conditions from infected areas such as skin, nail and hair [5,6]. Culturing of organisms from skin scraping was done on selective medium as Sabouraud Dextrose Agar for identification of dermatophytic species. For antifungal susceptibility testing these species were used after identifying them on cultural, morphological and biochemical characteristics [7]. Five antifungal drugs were used for testing. The microbroth dilution method was performed according to CLSI standards -M38-A [8].

Culture medium

Yeast Nitrogen Broth (YNB) supplemented with following composition was used.

YNB base 6.7 gm, Glucose 10.0 g, Distilled water 100 ml and adjusting the pH at 6.5. This medium was filtered, sterilized and used as basal medium (autoclaved). It was diluted to 1:10 with sterile distilled water just before use.

Antifungal agents

Antifungal drugs: Antifungal drugs used in this study were supplied from various firms, as follows: ketoconazole by Jansen Pharmaceuticals, fluconazole by Hydex Chemicals PvT. Ltd., terbinafine" named "Terbicip" produced by Cipla Ltd., and griseofulvin (also known as Grisovin, a proprietary name of Glaxo Laboratories. Itraconazole was used in its commercial formulation (Sri Pharma Care, INDIAMART). All drugs were dissolved in 100% dimethyl sulfoxide (Gibco) following the protocol of CLSI and were prepared in stock solutions of 1,000 µg/ml) and fluconazole in sterile distilled water, and kept at -200°C until used. They were subsequently prepared as stock solution and serial two fold dilutions were performed. Final concentrations ranged from 0.125 to 64 µg/mL for fluconazole, 0.03 to 16 µg/mL for ketoconazole, itraconazole and terbinafine, and 0.03 to 8 µg/mL for griseofulvin.

Preparation of inoculum

Testing was performed by a broth microdilution method following the recommendation of the CLSI M38-A. All the strains were obtained from the patient's samples of tinea infections. The species identification was based on morphological and biochemical characteristics and was used in inoculum preparation. In brief, stock inocula of dermatophytic stains were prepared from 7 to 14 day cultures grown on Sabouraud's dextrose agar (SDA) with chloramphenicol. After the appearance of the sufficient growth the fungal colonies were covered with 5 ml of sterile saline (0.9%), and the suspensions were made by gently probing the surface with the tip of a sterile Pasteur pipette. The resulting suspended mixture was withdrawn and transformed to a sterile tube. Heavy particles of the suspension, when present, were allowed to settle for 15 minutes at room temperature and the upper homogenous suspension was used for further testing. The suspensions were mixed with a vortex mixer for 15 seconds and adjusted with sterile normal saline to match the opacity of 0.5 McFarland's standard.

Turbidity standard for inoculum preparation

To standardize the inoculum density for a susceptibility test, a BaSO4 turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used. The inoculums size was adjusted to between 1.0 × 106 and 5.0 × 106 spores/ml by microscopic enumeration with a cell counting haemocytometer (Neubauer chamber). In some instance, where fungi do not readily produce conidia, small portion of the mycelial growth was harvested and gently homogenized in 2 ml of sterile saline using tenbroeck tissue grinder and resulting suspensions were adjusted to opacity of 0.5 McFarland standards by adding sterile saline. Inoculum quantification was made by counting microconidia in a hematocytometer and by plating 0.01 ml of suspensions in SDA. The plates were incubated at 28°C and were examined daily for the presence of fungal colonies before the test to check the viability of the fungus.

Test procedure

The tests were performed in a polysterene microtitre plates with flat bottom wells. By using a multichannel pipette the aliquots of 100 µl of two fold drug dilutions were inoculated into the wells. Then the microtitre plates were stored at -50°C in a deep freezer until used. The microplate was inoculated with 100µl fungal inoculum to maintain the dilutions with 0.5×10^4 to 5×10^4 spores ml^{-1}. The plates were incubated at 28°C for 7 days [9] for growth of the fungi. Growth and sterility control wells also maintained for each assay and all the tests were performed in duplicate. The highest dilution of the drug, which inhibited the fungal growth, was taken as the MIC. MIC50 was calculated by taking the drug concentration, where fifty percent of isolates are inhibited. Similarly MIC90 was noted with drug concentration where ninety percent of the isolates were inhibited The MIC values were noted basing on the rate of growth inhibition.

Results and Discussions

Antifungal susceptibility investigations

The fungal infections are not completely cured with antifungal drugs. The treatment is less successful, than that of bacterial infections because the fungal cells are eukaryotic and much more similar to human than the bacteria [10]. Many drugs that inhibit or kill fungi are therefore quite toxic for humans also. Moreover the fungal cells are equipped with a detoxifying system, which is able to modify many antibiotics; probably by hydroxylation [11]. Hence the antibiotics used to treat the fungal infection will remain fungistatic for a period of time and repeated usage of antibiotics are advised. The effective antifungal drugs may extract membrane sterols [12], or prevent their synthesis [13]. Most antifungal compounds target the formation or the function of ergosterol, an important component of the fungal cell membrane [14].

In the present study a total of 119 strains of dermatophytes belonging to 10 species were tested. All the strains were obtained from patient samples and were used in the tests. They were *T. rubrum* (n=40), *T. mentagrophytes* (n=19), *T. violaceum* (n=15), *M. gypseum* (n=12), *E. flocossum* (n=9), *M. audouinii* (n=8), *T. schoenleinii* (n=5), *M. canis* (n=5), T. *tonsurans* (n=4) and *T. verrucosum* (n=2).

Comparison of MICs of five antifungal agents

The minimum inhibitory concentrations (MIC 50 MIC 90) 0f griseofulvin, ketaconazole, fluconazole itraconazole and terbinafine are compared and presented in Table 1. The comparison of MIC values

Specie (No. of isolates)	MIC (µg/ml)	Griseofulvin	Ketoconazole	Fluconazole	Itraconazole	Terbinafine
T. rubrum (0)	MIC_{50}	1.28	0.24	1.28	0.24	0.005
	MIC_{90}	2.56	1.92	10.24	1.92	0.04
	Range	0.16-5.12	0.01-3.84	0.16-20.48	0.03-3.84	0.001-0.08
T. mentagrophytes (14)	MIC_{50}	1.28	0.12	1.28	0.24	0.06
	MIC_{90}	2.56	0.24	10.24	0.96	0.08
	Range	0.32-5.12	0.01-0.96	0.08-20.48	0.03-1.92	0.002-0.16
T. violaceum (19)	MIC_{50}	1.28	0.48	2.56	0.12	0.01
	MIC_{90}	2.56	0.96	5.12	0.48	0.04
	Range	0.32-5.12	0.03-1.92	0.16-10.24	0.01-0.96	0.001-0.08
M. gypseum (12)	MIC_{50}	1.28	0.96	10.24	0.12	0.08
	MIC_{90}	2.56	1.92	20.28	0.48	0.32
	Range	0.64-5.12	0.01-3.84	0.16-40.96	0.03-0.96	0.005-0.64
E. flocossum (9)	MIC_{50}	1.28	0.48	2.56	0.06	0.002
	MIC_{90}	2.56	0.96	5.12	0.24	0.01
	Range	0.32-5.12	0.03-1.92	0.64-10.24	0.03-0.48	0.001-0.04
M. audouinii (8)	MIC_{50}	1.28	0.12	2.56	0.12	0.02
	MIC_{90}	2.56	0.96	5.12	0.48	0.08
	Range	0.32-5.12	0.03-1.92	0.32-10.24	0.03-0.96	0.005-0.16
T. schoenleinii (5)	MIC_{50}	0.64	0.24	2.56	0.24	0.02
	MIC_{90}	1.28	0.48	5.12	0.48	0.04
	Range	0.32-2.56	0.06-0.96	0.32-10.24	0.12-0.96	0.01-0.08
M. canis (5)	MIC_{50}	1.28	0.24	5.12	0.96	0.005
	MIC_{90}	5.12	0.48	10.24	1.92	0.01
	Range	0.64-5.12	0.06-0.48	0.64-20.48	0.24-3.84	0.002-0.01
T. tonsurans (4)	MIC_{50}	1.28	0.06	2.56	1.92	0.01
	MIC_{90}	2.56	0.12	5.12	3.84	0.02
	Range	0.64-5.12	0.01-0.48	0.16-20.48	0.48-7.68	0.005-0.04
T. verrucosum (2)	MIC_{50}	0.64	0.03	2.56	0.24	0.04
	MIC_{90}	1.28	0.12	5.12	0.96	0.08
	Range	0.32-1.28	0.03-0.12	0.32-5.12	0.12-0.92	0.02-0.08
Total no. of Isolates (119)	MIC_{50}	1.28	0.24	2.56	1.28	0.02
	MIC_{90}	2.56	1.92	10.24	2.5	0.32
	Range	0.32-5.12	0.01-3.84	0.08-20.45	0.32-5.12	0.001-0.64

Table 1: Comparison of MICs of five antifungal agents.

is used in determining the efficacy and the dosage of drug for the treatment of dermatophytosis. The data presented in Table 1 is critically analyzed.

The analysis revealed that griseofulvin exhibited MIC 50 at 1.28 µg/ml for *T. rubrum, T. mentagrophytes, T. violaceum, M. gypseum, E. flocossum, M. audouinii , M. canis and T. tonsurans;* at 0.64 µg/ml for *T. schoenleinii* and *T. verrucosum.*

Ketoconazole showed MIC 50 at 0.24 µg/ml for *T. rubrum, T. schoenleinii* and *M. canis*; at 0.12 µg/ml for *T. mentagrophytes* and *M. audouinii*; at 0.48 µg/ml for *T. violaceum and E. floccosum*; at 0.96 µg/ml for *M. gypseum*; at 0.06 µg/ml for *T. tonsurans*; at 0.03 µg/ml for *T. verrucosum.*

Fluconazole showed MIC 50 at 1.28 µg/ml for *T. rubrum* and *T. mentagrophytes*; at 2.56 µg/ml for *T. violaceum, E. flocossum, M. audouinii, T. schoenleinii*, *T. tonsurans* and *T. verrucosum*; at 10.24 µg/ml for *M. gypseum*, and at 5.12 µg/ml for *M. canis*.

Itraconazole exhibited MIC 50 at 0.24 µg/ml for *T. rubrum*, *T. mentagrophytes*, *T. schoenleinii* and *T. verrucosum;* at 0.12 µg/ml for *T. violaceum, M. gypseum* and *M. audouinii*; at 0.06 µg/ml for *E. flocossum* and at 0.09 µg/ml for *M. canis*; at 1.92 µg/ml for *T. tonsurans.*

Terbinafine showed MIC 50 at 0.005 µg/ml for *T. rubrum* and *M. canis*; at 0.06 µg/ml for *T. mentagrophytes*; at 0.01 µg/ml for *T. violaceum* and *T. tonsurans*; at 0.*08* µg/ml for *M. gypseum*, at 0.002 µg/ml for *E. flocossum*; at 0.02 µg/ml for, *M. audouinii*, and *T. schoenleinii*; at 0.04 µg/ml for *T. verrucosum*.

A critical analysis of Table 1, showed the MIC 90 of griseofulvin at 2.56 µg/ml for *T. rubrum, T. mentagrophytes, T. violaceum, M. gypseum, E. flocossum, M. audouinii* and *T. tonsurans*; at 1.28 µg/ml for *T. schoenleinii and T. verrucosum*; at 5.12 µg/ml for *M .canis*.

Ketoconazole exhibited the MIC 90 at 1.92 µg/ml for *T. rubrum* and *M. gypseum*; at 0.24 µg/ml for *T. mentagrophytes*; at 0.96 µg/ml for *T. violaceum, E. flocossum* and *M. audouinii*; at 1.92 µg/ml for *M. gypseum*; at 0.48 µg/ml for *T. schoenleinii* and *M. canis*; at 0.12 µg/ml for

T. tonsurans and *T. verrucosum*.

Fluconazole showed MIC 90 at 10.24 µg/ml for *T. rubrum*, *T. mentagrophytes* and *M. canis*; at 20.48 µg/ml for *M. gypseum* and 5.12 µg/ml for *T. violaceum, E. flocossum, M. audouinii, T. schoenleinii, T. tonsurans* and *T. verrucosum*.

Itraconazole exhibited the MIC 90 at 1.92 µg/ml for *T. rubrum* and *M. canis*; at 0.96 µg/ml for *T. mentagrophytes* and *T. verrucosum*; at 0.48 µg/ml for *T. violaceum*, *M. gypseum*, *M. audouinii* and *T. schoenleinii*; at 0.24 µg/ml for *E. flocossum* and at 3.84 µg/ml for *T. tonsurans*.

Terbinafine showed the MIC 90 at 0.04 µg/ml for *T. rubrum T. violaceum* and *T. schoenleinii*; at 0.08 µg/ml for *T. mentagrophytes, M. audouinii* and *T. verrucosum*; at 0.32 µg/ml for *M. gypseum*; at 0.01 µg/ml for *E. flocossum* and *M. canis*; at 0.02 µg/ml for *T. tonsurans*.

The MIC ranges for all the 119 isolates of dermatophytes tested for antifungal susceptibility showed that terbinafine had the lowest MIC range of 0.001 to 0.64 µg/ml followed by ketoconazole at a MIC range of 0.01-3.84 µg/ml. The itraconazole showed a MIC range of 0.082-20.45 µg/ml whereas the griseofulvin and fluconazole showed a highest MIC range of 0.32-5.12 µg/ml. The MIC 50 of Terbinafine was low at 0.02 µg/ml followed by Ketaconazole 0.24 µg/ml. The MIC 50 of itraconazole and griseofulvin was1.28 µg/ml .The highest MIC 50 with 2.56 µg/ml was recorded for Fluconazole. The MIC90 of terbinafine was low at 0.32 µg/ml followed by Ketaconazole with 1.92 µg/ml. The MIC 90 Itraconazole was 2.50 µg/ml and for griseofulvin it was 2.56 µg/ml. The highest MIC 90 of flucanozole was at 10.24 µg/ml.

In our study, we observed that terbinafine had the lowest MIC values compared to ketoconazole, itraconazole, griseofulvin and fluconazole. Our observations regarding the efficacy of terbinafine are corroborating with the study reports of other workers. Gupta AK, (2003) [15] in their study report on '*In vitro* susceptibility testing of ciclopirox, terbinafine, ketoconazole and itraconazole against dermatophytes and nondermatophytes' stated that terbinafine is extremely potent against dermatophytes.

The other study reports also correlated with our study, in which, the MICs of terbinafine and itraconazole were significantly higher than fluconazole. The MICs of ketoconazole and griseofulvin varied among strains [16]. Favre et al., (2003), [17] reported that allylamine terbinafine was the most potent agent against some dermotophytes spp. Even the terbinafine was proved as an extremely potent antifungal drug against Trichophyton spp [18] followed by itraconozole [19]. But in our study report the MIC50 of Terbinafine was low at 0.02 µg/ml followed by ketoconazole 0.24 µg/ml. (0.04 µg/mL). This variation in our results may be due to species-specific susceptibility against antifungal drugs.

Conclusion

In conclusion, it may be useful to undertake periodical screening programs to detect the antifungal susceptibility of newer antifungal agents. Our data on the antifungal susceptibility of dermatophyte isolates may contribute to a choice of antifungal treatment to ringworm infections. Terbinafine is considered as most potent drug followed by ketoconazole. But still the efficacy of ketoconazole drug was totally dependent upon the variation of causative dermatophytic strains of particular tinea infections. We consider that our study on the antifungal susceptibility of dermatophytes can be beneficial for investigation of *in vitro* resistance of dermatophytic species, and for management of cases clinically unresponsive to treatmenT.

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