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A Study of *invitro* Antifungal Susceptibility Patterns of Dermatophytic Fungi at a Tertiary Care Center in South India

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A B S T R A C T

Dermatophytes are keratinophilic and keratinolytic fungi. They are responsible for dermatophytosis which are superficial mycosis. Any clinical diagnosis needs to be supported by laboratory diagnosis. Culture is a necessary adjunct to direct microscopic examination for definitive identification of etiological agent and in many instances the choice of therapy depends upon the specific identification of the invasive mould. Standardization of in vitro susceptibility testing provides consistent and reproducible data that may predict clinical response when used in conjunction with individual patient risk factors. An ideal antifungal drug should have broad spectrum activity, should be effective in vivo, minimum side effects and there should be no drug resistance and minimum side effects. All clinically diagnosed cases of dermatophytosis in all age groups and of both sexes, attending the Outpatient Department of Dermatology and Venereology were taken for the study. After isolation of the fungi from clinical samples, isolates were inoculated in fungal media and antifungal susceptibility testing was done. Antifungals tested were Ketaconazole, Terbinafine, Clotrimazole and Sertaconazole. Range of MIC values of ketoconazole for *T. rubrum* (33), *T.mentagrophytes (*14), *T. tonsurans* (7) and *M.gypseum* were 0.03-4µg/ml, 0.03-1µg/ml, 0.125-1µg/ml and 0.12-0.5µg/ml respectively. In remaining 6 isolates of *T.mentagrophytes* 3 had MIC of 2µg/ml and other 3 isolates had MIC of 3µg/ml for ketoconazole, which is above the normal range (0.03-1µg/ml) and 1 isolate of *T.tonsurans* had MIC of 2µg/ml. Range of MIC values of Terbinafine for T. rubrum, T.mentagrophytes, T. tonsuranse and M. gypseum were 0.03-0.5µg/ml, 0.03-1µg/ml, 0.03-0.06µg/ml and 0.15µg/ml respectively. Range of MIC value of Clotrimazole for *T. rubrum*, *T.mentagrophytes*, *T. tonsurans* and *M. gypseum*was 0.031-0.5µg/ml. Range of MIC value of sertaconazole for *T.rubrum*was 0.03-0.62µg/ml and for *T.mentagrophytes*, *T. tonsurans* and *M. gypseum* was 0.03-0.125µg/ml. Majority of the dermatophyte isolate had MIC within normal range. Few of the isolates of *T.mentagrophytes* and *T.tonsurans* showed higher MIC. Periodic evaluation of Antifungal Susceptibility Testing is necessary especially in immune suppressive illness and in chronic dermatophytosis to find out antifungal resistance which would help the clinicians to select appropriate antifungal agents.

Introduction

Dermatophytes are keratinophilic and keratinolytic fungi. They are responsible for dermatophytosis which are superficial mycosis affecting skin (examples for dermatophytosis include, tinea corporis, tinea cruris, tinea pedis). hair (tinea capitis), beard (tinea barbae) nails (onychomycosis or tinea unguium). Dermatophytosis is a nonfatal disease except in extremely rare cases of Hadida and Schousboe's dermatophytic disease.[1]

Dermatophytes are a group of closely related organisms that can use keratin as a nitrogen source. On the basis of clinical, morphologic and microscopic characteristics three genera are recognized as Dermatophytes; Trichophyton, Microsporum and Epidermophyton.

Any clinical diagnosis needs to be supported by laboratory diagnosis. Culture is a necessary adjunct to direct microscopic examination for definitive identification of etiological agent and in many instances the choice of therapy depends upon the specific identification of the invasive mould[2]. This is especially important in nail and skin infection, often caused by non-dermatophytic filamentous fungi, which are often resistant to usual dosage of the therapy used for dermatophytic infections. Identification of fungal hyphae in the macerated skin of the web of toes may be difficult due to superadded bacterial infection. Before starting treatment for dermatophytosis, it is essential to establish the diagnosis of the disease, so that specific therapeutic modalities can be monitored during the course of the treatment.[2]

Now effective drugs are available for chemotherapy of fungal infections and further modalities are being developed for management of the same. Standardization of in vitro susceptibility testing provides consistent and reproducible data that may predict clinical response when used in conjunction with individual patient risk factors. An ideal antifungal drug should have broad spectrum activity, should be effective in vivo, minimum side effects and there should be no drug resistance and minimum side effects.

The main aim and objectives of this study to perform antifungal susceptibility testing of the fungal isolates by microbroth dilution method.

Materials and Methods

The present study of dermatophytosis was carried out in the Department of Microbiology at a tertiary care Hospital and Medical Research Centre in south India, over a Period of one year. All clinically diagnosed cases of dermatophytosis in all age groups and of both sexes, attending the Outpatient Department of Dermatology and Venereology were taken for the study.

Inclusion Criteria

All skin, hair and nail samples from clinically suspected cases of dermatophytosis.

Exclusion Criteria

Patients who are already on treatment for dermatophytosis.

Culture

The specimens collected were inoculated on to Sabourauds Dextrose agar containing Chloramphenicol (50mg/l) and Cycloheximide (500mg/l); irrespective of demonstration of fungal elements on KOH mount. Each sample was inoculated into a pair of tubes. One tube with antibiotic and other without antibiotic and were incubated at 27^0C. The cultures were examined daily for a period of 4 weeks.

Slopes showing no growth for4 weeks were discarded. If growth was obtained on Sabourauds Dextrose agar, identification was made based on colony morphology, microscopic appearance and other relevant tests. The isolates were inoculated on potato dextrose agar for better conidiation.

Macroscopic Examination of Culture

The growth on Sabourauds dextrose agar was observed to study the colony morphology, the color of the surface, the reverse of the colony, the texture of the surface, the topography and the rate of growth.

Antifungal Susceptibility Testing For Dermatophytes

Microbroth Dilution Method

Requirements

Sterile test tubes for drug dilution / inoculum preparation

Sterile disposable microtitre plates

Sterile Micro pipette / sterile tips /Gloves / disposable face masks

Whatmann filter paper no 40.

Antifungals (Ketoconazole, Clotrimazole, Terbinafine, Sertaconazole)

Solvents (DMSO, Ethanol, Methanol)

Medium

RPMI 1640 with glutamine, without bicarbonatein MOPS (3N-Morpholino propanesulphonic acid), buffer sterilized by membrane filtration.

Anti Fungal Stock Solution

10ml stock solution prepared for each drug.

For water insoluble drugs-diluent DMSO Solvents

DMSO (Ketoconazole)

Methanol (Sertaconazole)

Ethanol (Terbinafine, Clotrimazole)

Drug dilutions

Antifungal stock solution preparation

$$\text{Weight (mg)} = \text{volume (mL)} \times \text{desired concentration (mg/mL)} \;/\; \text{Antifungal potency}$$

For example, to prepare for a broth microdilution test series containing a water insoluble

Drug that can be dissolved in respective solvents, for which the highest desired test concentration is 1600µg/ml, first weigh 16.0 mg (assuming 100% potency) of antifungal powder and dissolve in 10ml of solvent. This will provide a stock solution at1, 600µg/ml.

Label 9 tubes as 3–11.

Add appropriate amounts of DMSO to each tube as follows:

Add 0.5 mL of DMSO to tubes 3, 6, and 9

Add 0.75 mL of DMSO to tubes 4, 7, and 10

Add 1.75 mL of DMSO to tubes 5, 8, and 11

Label the stock solution tube (1,600 µg/mL) as tube 2
Transfer from tube 2, 0.5 mL to tube 3 and 0.25 mL to tubes 4 and 5

Transfer from tube 5, 0.5 mL to tube 6 and

0.25 mL to tubes 7 and 8

Transfer from tube 8, 0.5 mL to tube 9 and 0.25 mL to tubes 10 and 11

Finally, discard 1 mL from tube 11

Notice that when dilutions have been prepared, all the tubes contain 1 mL

Dilution of drug used were between 32µg/ml to 0.0625 µg/ml, {32, 16, 8, 4, 2,

1, 0.5, 0.25, 0.125, 0.0625}

Drug Dilution

To prepare 5ml volume of antifungal agent first pipette 4.9 ml volumes of RPMI1640 medium into each of 10 sterile test tubes. Now, add0.1ml solvent in tubes (sterility control and growth control). Add 0.1ml of the corresponding drug dilution to each of the tubes. These volumes were adjusted according to the total No. of test required. Because there will be 1:2 dilution of the drug when combined with the inoculum, the working antifungal solutions are twofold more concentrated than the final concentration.

Final concentration after inoculation is {16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 0.031µg/ml}

Inoculum Preparation

7-15 days old cultures grown on PDA at 25° C was used. Mature colonies were covered with 10ml of sterile saline (0.85 %). Growth scraped by sterile Pasteur pipette. Heavy particles were allowed to settle for 15-20minutes at room temperature. Supernatant was mixed with a vortex for 15 seconds. Then it was filtered through what mann filter paper no 40. Turbidity was adjusted spectrophoto-metrically to 530nm 65 -70%absorbance. Each suspension was diluted 1: 50 in RPMI 1640 medium. The growth control wells contain 0.1ml of the corresponding diluted inoculum suspension and 0.1ml of the drug diluent without antifungal agents.

Test Procedure

Test was performed in sterile microtiter plates. Aliquots of 100µls of drug dilutions were inoculated in 1-10 microtiter wells. Add 100 µl of inoculum into each well from 1to 12. Growth control -tube 12 with inoculum and without antifungal drug.

Incubation

All microdilution trays were incubated at 28ºC without agitation.

Reading Results

Lowest concentration of the drug which permitted no macroscopically visible growth after 7 days was taken as MIC (Minimum Inhibitory Concentration).

MIC results recorded in µg/ml. (NCCLS M-38A)

Quality Control

Trichophyton mentagrophytes ATCC 9533 was procured from Tertiary care hospital from Maharashtra.

Antifungals were obtained from

Setraconazole, Ketoconazole - NOVARTIS Mumbai

Terbinafine - MICROLABS Bangalore

Clotrimazole - FDC Ltd Mumbai

Results and Discussion

Out of 32 clinical cases of *Tinea cruris*, 21 dermatophytes were isolated *T.rubrum* was isolated from 13 cases T. *mentagrophytes* was isolated from 6 samples, *T tonsurans* was

isolated from 2 samples.

The present study included 3 cases from Tinea capitis cases, out of which *T. Tonsurans* was isolated from 1 sample.

Out of 20 cases of Tinea unguium*T. rubrum*, *T. mentagrophytes*, and *T. tonsurans*, were isolated from 2, 2, and 1 clinical samples respectively.

Out of 12 samples from T.pedis cases, *T. rubrum* was isolated from 5, *T. mentagrophytes* from 4, *M. gypseum* from 1 clinical sample.

Tinea manuum was least common clinical type of dermatophytosis in our study with incidence of 2 cases. From 1 sample T. *rubrum* was isolated.

Out of 31 clinical cases from Tinea corporis *T. rubrum* was the commonest isolate with incidence of 12. *T mentagrophyte* was isolated from 8 clinical samples. *T. tonsurans* was isolated from 4 samples. *M. gypseum* from 1 clinical sample (Tables 1 and 2)

Out of 100 clinical samples 77 were culture positive and 23 were culture negative

Out of 100 clinical samples 58 samples were culture positive and KOH positive. 19 samples were Culture positive and KOH negative. 18 samples were Culture as well as KOH negative. 5 samples were KOH positive and culture negative.

In present study antifungal sensitivity was determined for *T. rubrum*, *T.mentagrophytes*, *T. tonsurans* and *M. gypseum.* Antifungals tested were Ketaconazole, Terbinafine, Clotrimazole and Sertaconazole.

Range of MIC values of ketoconazole for *T. rubrum* (33), *T.mentagrophytes (*14), *T. tonsurans* (7) and *M.gypseum* were 0.03-4µg/ml, 0.03-1µg/ml, 0.125-1µg/ml and 0.12-0.5µg/ml respectively.

In remaining 6 isolates of *T.mentagrophytes* 3 had MIC of 2µg/ml and other 3 isolates had MIC of 3µg/ml for ketoconazole, which is above the normal range (0.03-1µg/ml) and 1 isolate of *T.tonsurans* had MIC of 2µg/ml.

Range of MIC values of Terbinafine for T. rubrum, T.mentagrophytes, T. tonsuranse and M. gypseum were 0.03-0.5µg/ml, 0.03-1µg/ml, 0.03-0.06µg/ml and 0.15µg/ml respectively.

Range of MIC value of Clotrimazole for *T. rubrum*, *T.mentagrophytes*, *T. tonsurans* and *M. gypseum* was 0.031-0.5µg/ml.

Range of MIC value of sertaconazole for *T.rubrum* was 0.03-0.62µg/ml and for *T.mentagrophytes*, *T. tonsurans* and *M. gypseum* was 0.03-0.125µg/ml. (Table 3)

All the above mentioned MIC values of Ketoconazole, Terbinafine, Clotrimazole and Sertaconazole was compared with the standard *T.mentagrophytes ATCC* 9533

MIC value of *T.mentagrophytes ATCC:* Ketoconazole – 0.06µg/ml

Terbinafine - <0.03µg/ml

Clotrimazole – 0.03µg/ml

In present study 100 clinically diagnosed cases of Dermatophytosis were studied. Of them 77 were skin scrapings, 20 were nail clippings and 3 were hair stubs. Out of these samples, dermatophytes were isolated in 63 cases. Among 63 dermatophytes isolated, *T.rubrum* was the commonest species (33) followed by *T.mentagrophytes* (20). *T.tonsurans* was isolated from (8), *M.gypseum* in (2), other than dermatophytes *Penicillium spp* was isolated in 3 cases, *A.niger* in 7, *Acremonium* in 3 and

Curvilaria in 4. No fungal growth was seen in 22 clinical samples.

The overall isolation rate of dermatophytes was 63%. Tinea cruris accounted for maximum number of cases. Antifungal susceptibility was carried out for all the dermatophytes isolated (63). Microbroth dilution method was used to determine the MIC for Ketoconazole, Terbinafine, Clotrimazole, and Sertaconazole according to NCCLS (CLSI) M38A (2007) document for antifungal susceptibility testing for filamentous fungi with some modifications. The CLSI approved guidelines recommended separation of the fungal structures (hyphae and conidia) through sedimentation for 15 to 20 min and use of the upper part of the suspension for susceptibility testing.[3]. The separation of hyphae and conidia a crucial step for the determination of MICs for dermatophytes. Whatman no 40 was used to filter the inoculum as it retains the hyphal fragment and permits the passage of only microconidia of dermatophytes.[4]

These antifungals were preferred mainly because these were the most commonly used topical and systemic drugs by the clinicians in the treatment of dermatophytosis. Sertaconazole is a newer azole available for topical use in the treatment of dermatophytosis. Ketoconazole is costly when compared to other drugs.

Terbinafine

In the present study it was found that for all the species isolated, Terbinafine had low MIC range and this is comparable with most of the other studies like Norris *et al.*,[5], Ghannoum *et al.*,[6].

The results of Santos *et al.*, and Carrillo-Mun˜oz *et al.*,[7] is similar to that of the present study. As few isolates in present study showed MIC of <0.03µg/ml. (Table 4)

MIC of *T.mentagrophytes* ATCC 9533 which was used as control also showed within the range as in the present study

Ketoconazole

Ketoconazole has higher MIC in a study done by Cetinkaya *et al.*, [9] when compared to the present study this could because of different size of the inoculum used or incubated for longer duration.

The present study had similar results as those of Araujo *et al.*,[8,] Santos *et al.*,[4], and Pujol *et al.*,[10]. Siqueira *et al.*, [11]showed lower MIC than the present study. Few isolates of *T.tonsurans* and *T.mentagrophytes* in the present study showed higher MIC value than the other isolates (Table 5). *T.mentagrophytes* ATCC 9533 which was used as control showed MIC 0.06µg/ml

Table.1 Various Clinical Types of Dermatophytosis

Dermatophytosis	Number
Tinea cruris	32
Tinea capitis	3
Tinea unguium	20
Tinea pedis	12
Tinea manuum	2
Tinea corporis	31

Fig.1 Mode of Processing the Sample

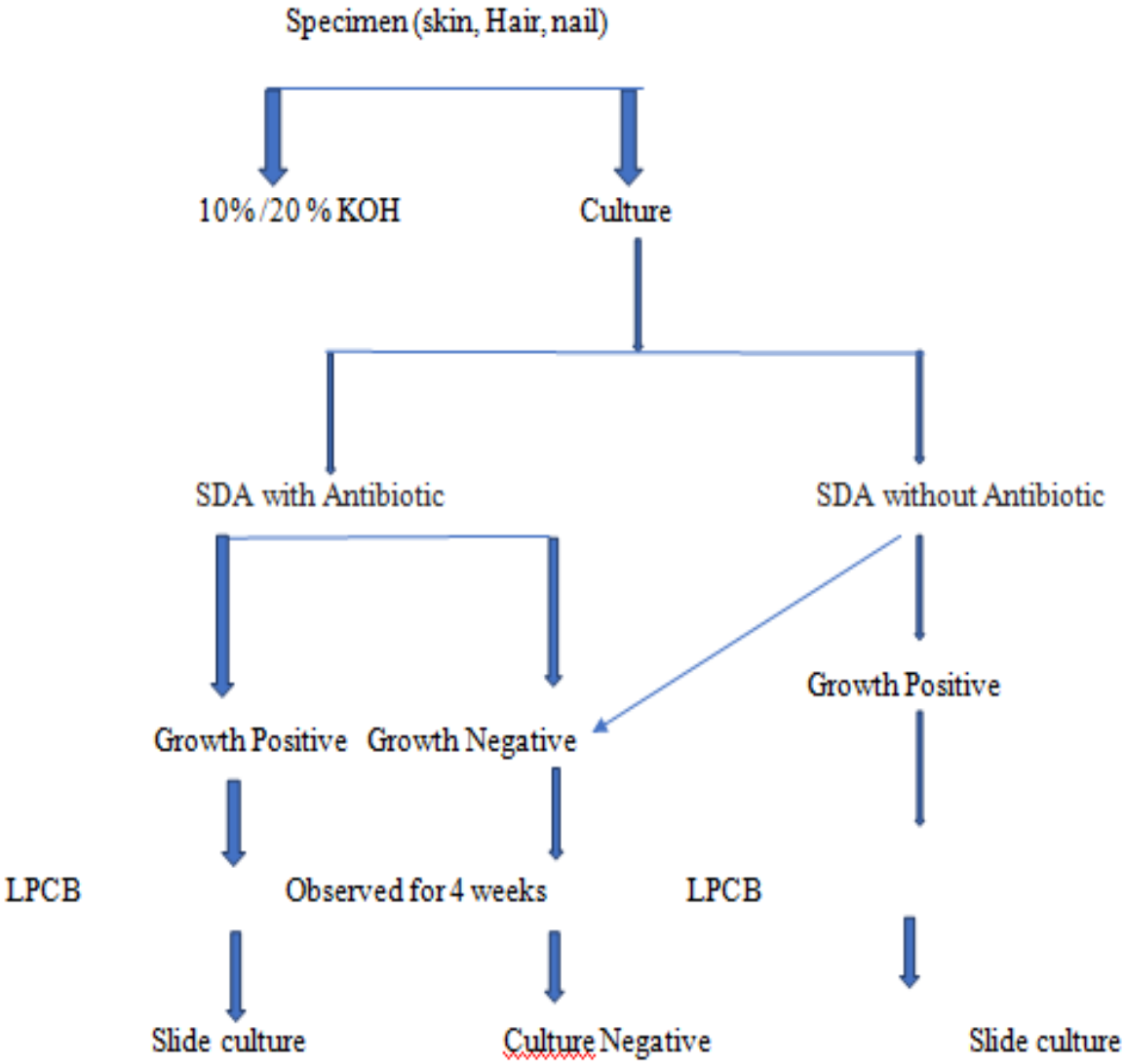

Table.2 Incidence of Various Dermatophytes isolated

Isolates	Number
Trichophyton rubrum	33
Trichophyton mentagrophytes	20
Trichophyton tonsurans	8
Microsporumgypseum	2

Table.3 Minimum Inhibitory Concentration for various antifungal agents

Dermatophyte	MIC Value Range in µg/ml			
	Ketaconazole	**Terbinafine**	**Clotrimazole**	**Sertaconazole**
T. rubrum	0.03-4	0.03-0.5	0.031-0.5	0.03-0.62
T.mentagrophytes	0.03-1	0.03-1	0.031-0.5	0.03-0.125
T. tonsurans	0.125-1	0.03-0.06	0.031-0.5	0.03-0.125
M. gypseum	0.12-0.5	0.15	0.031-0.5	0.03-0.125

Table.4 MIC values for Terbinafine from various studies

Studies	Species	MIC range µg/ml
Santos et al (2005)[4]	*Trichophyton sps*	<0.031
Ghannoum et al (2004)[6]	*Trichophyton sps*	0.001–0.5
Araujo et al (2009)[8]	*T. rubrum* *T.mentagrophytes* *M.gypseum*	0.03-0.5 0.03-1 0.15
Norris et al (1999)[5]	*Trichophyton sps*	<0.06
Carrillo-Mun˜oz et al (1997)[7]	All dermatophytes	0.03
Present study	*T.rubrum* *T.mentagrophytes* *T.tonsurans* *M .gypseum*	0.03-0.5 0.03-1 0.03-0.06 0.15

Table.5 MIC values for Ketoconazole from various studies

Studies	Species	MIC range µg/ml
Santos et al (2005)[4]	*T. rubrum*	0.0625–2.0
Pujol et al (2002)[10]	All dermatophytes	0.03-2
Araujo et al (2009)[8]	*T. rubrum* *T.mentagrophyte*	0.03-4 0.03-1
Cetinkaya et al (2005)[9]	*T.rubrum* *T.mentagrophytes* *T.tonsurans*	0.03-8 0.25-2 0.12-0.25
Siqueira et al (2008)[11]	*Trichophyton sps*	<0.03-0.5
Present study	*T.rubrum* *T.mentagrophytes* *T.tonsurans* *M .gypseum*	0.03-4 0.03-1 0.12-1 0.12-0.5

Table.6 MIC values of Clotrimazole from various study

Studies	Species	MIC µg/ml
Santos et al (2006)[4]	All dermatophytes	0.03-0.5
B. Favre et al (2003)[12]	All dermatophytes	0.083
Present study	All dermatophytes	0.03-0.5

Table.7 Various studies showing MIC values

Studies	Species	MIC µg/ml
Carrillo-Mun˜oz *et al* (1997)[7]	*T.rubrum* *T.mentagrophytes* *T .tonsurans* *M.gypseum*	0.03-0.62 0.03-0.12 0.03-0.12 0.3-1.2
Palacı´n *et al* (1992)[13]	All dermatophytes	0.24
Present study	*T.rubrum* *T.mentagrophytes* *T .tonsurans* *M.gypseum*	0.03-0.62 0.03-0.12 0.03-0.12 0.03-0.12

Clotrimazole

Favre *et al.*,[12] showed lower MIC value when compared to present study whereas the results of Santos *et al.*,[4] was similar (Table 6).

Sertaconazole

MIC of Sertaconazole from various studies Palacin *et al.*,[13] and Carrillo Munoz *et al.*,[7] had similar results as the present study (Table 7).
MIC of *T.mentagrophytes ATCC 9533*: 0.06µg/ml

In the present study Terbinafine was found to be more potent when compared to other drugs with lower MIC values (<0.03µg/ml) followed by Sertaconazole and Clotrimazole. Terbinafine >Sertaconazole> Clotrimazole > Ketoconazole. Ketoconazole had higher MIC and also some isolates had MIC more than the normal range. In recent years several studies of in vitro susceptibility of dermatophytes have been done and the results have shown considerable variations. This variability is probably due to important methodological differences like preparation of inoculum, incubation at different temperatures, no of days of incubation is different among the laboratories. Dermatophytosis is the most common type of cutaneous fungal infection. It is very common in our country with several contributing factors like hot humid climate, poor hygiene, increased outdoor activities, occupational trauma and immunosuppression.

The incidence of Dermatophytosis is increasing in India due to widespread and indiscriminate use of corticosteroids and antifungal agents without appropriate microbiological investigations. Dermatophytes isolated included predominately *Trichophyton* species, of which *T.rubrum*was the commonest dermatophyte isolated. *T.mentagrophytes*, *T.tonsurans*, *M.gypseum* were other isolates from clinical samples.

Majority of the dermatophyte isolate had MIC within normal range. Few of the isolates of *T.mentagrophytes* and *T.tonsurans* showed higher MIC. Periodic evaluation of Antifungal Susceptibility Testing is necessary especially

in immune suppressive illness and in chronic dermatophytosis to find out antifungal resistance which would help the clinicians to select appropriate antifungal agents.

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