Research Article

To determine antifungal susceptibility of dermatophyte isolates in a tertiary care hospital using microdilution method: A prospective cohort study

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ABSTRACT

Aim: The aim of the study was to standardize *in vitro* antifungal susceptibility testing by microbroth dilution method to find out the minimum inhibitory concentration (MIC) of fluconazole, ketoconazole, itraconazole, terbinafine, and griseofulvin fungal isolates of skin, hair, and nail. **Materials and Mehods:** Various samples were collected from patients with clinically diagnosed dermatophytosis. Skin scrapings, hair, and nail were collected from 170 patients. **Results:** The antifungal drugs such as fluconazole (0.5–0.16 µg/ml), ketoconazole (0.03–0.5 µg/ml), itraconazole (0.007–0.25 µg/ml), terbinafine (32–0.0313 µl/ml), and griseofulvin (0.03–0.25 µg/ml) these water-insoluble drugs were incorporated in dissolved in dimethyl sulfoxide. The MIC range, MIC 50, and MIC 90 for the drug griseofulvin were found to be 0.03–0.25, 0.06, and 0.12, respectively, for ketoconazole were found to be 0.03–0.5, 0.12, and 0.5, respectively, for the drug fluconazole were found to be 1–32, and 1, respectively, itraconazole were found to be 0.007–0.06, 0.03, and 0.06, respectively, and for terbinafine were found to be 0.007–0.06, 0.015, and 0, respectively. This technique was found to be reliable, cost-effective, and easy to perform with consistent results. **Conclusion:** Further, results concluded that the itraconazole showed a higher MIC value when compared to other antifungal drugs.

Keywords: Antifungal susceptibility testing, fluconazole, griseofulvin, itraconazole, ketoconazole, microbroth dilution method, minimum inhibitory concentration, terbinafine

Introduction

Dermatophytes are a group of fungi affecting keratinized portion of skin and its appendages, hair, and nail. Dermatophytes comprise three genera trichophyton, epidermophyton, and microsporum.[1,2] The incidence of dermatophytosis has been increasing.[1] Topical antifungals are used if the lesions are smaller and systemic antifungals are administered in case of extensive lesions and the course usually is for several weeks. Griseofulvin, ketoconazole, fluconazole, itraconazole, and terbinafine are among the commonly used agents.[3]

Determining antifungal susceptibility among dermatophytes is challenging, especially when it comes to standardization of inoculum, reading of the results as the endpoints are not very clear, variations in optimum temperature, duration of incubation, etc.[3] Although several methods have been tried for testing antifungal susceptibility among dermatophytes, broth microdilution method is recommended by Clinical and Laboratory Standards Institute (CLSI) in M38-A document and is widely accepted.[4]

It has been observed that several times in spite of prolonged administration of antifungals tinea infection fails to get completely cured and resistance to antifungals has been reported in dermatophytes. This necessitates testing of dermatophyte isolates for susceptibility to commonly used antifungals. This helps in choosing not only an effective antifungal but also provides a choice regarding safety, economy, and ease of administration. Antimicrobial resistance is known to vary in different geographical areas and during different time period at the same geographical area.[2,5] There is a paucity of literature on the antifungal susceptibility patterns at our geographical region. This study was conducted to determine minimum inhibitory concentrations (MICs) for five commonly used antifungal agents among the common clinical dermatophyte isolates in this geographical area.

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Methodology

Type of study - It was a hospital-based prevalence study.

Study period

The present study was conducted in the Department of Microbiology at Government Stanley Medical College and Hospital, Chennai, and over a period of 1 year from May 2008 to June 2009.

Sample specifications

Skin scrapings, hair, and nail were collected from 170 patients who attended the mycology section in the Dermatology Outpatient Department at Stanley Medical College and Hospital Chennai.

Inclusion criteria

All consenting patients with clinically diagnosed dermatophytosis irrespective of age and sex who were not undergoing treatment for the same were included in the study.

Exclusion criteria

All patients with ringworm infection and who were on pharmacological treatment were excluded from the study.

Antifungal susceptibility testing[4]

It was done by broth microdilution method as per CLSI M38-A method. Susceptibility patterns of the dermatophyte isolates were evaluated for fluconazole, ketoconazole, itraconazole, griseofulvin, and terbinafine.

Medium

RPMI 1640 with glutamine, without bicarbonate in 3N-morpholinopropanesulfonic acid buffer was sterilized by membrane filtration.

Antifungal stock solution

About 5 ml stock solutions were prepared for each drug. For water-soluble drugs (fluconazole),

2-fold dilutions were used. For water-insoluble drugs, dimethyl sulfoxide (DMSO) was used as diluent.

Drug dilution

To prepare 5 ml volumes of antifungal agent, first 4.9 ml volumes of RPMI 1640 medium were pipetted into each of 10 sterile test tubes. Now, using a single pipette, 0.1 ml of DMSO alone was added to one 4.9 ml lot of medium (control medium), then 0.1 ml of lowest (3.13 µg/ml) drug concentration in DMSO, then 0.1 ml of the 6.25 µg/ml concentration, and it was continued in sequence up the concentration series, each time adding 0.1 ml volumes to 4.9 ml medium. These volumes were adjusted according to the total number of test required. Because there will be 1:2 dilution of the drug when combined with the inoculum, the working antifungal solutions are 2-fold more concentrated than the final concentration.

Inoculum preparation

7–15-day-old cultures grown on SDA at 25°C were used. Mature colonies were covered with 10 ml of sterile saline (0.85%). Growth was scraped by sterile Pasteur pipette. Heavy particles allowed to settle for 15–20 min at room temperature. Supernatant was mixed with a vortex for 15 s. Turbidity of supernatant was adjusted spectrophotometrically to 530 nm 65–70% absorbance. Each suspension was diluted 1:50 in RPMI 1640.

Inoculating RPMI-1640 medium

Each well was inoculated on the day of test with 0.1 ml of ×2 inoculum suspension. This step will dilute the drug concentration, inoculum densities, and solvent used to the final desired test concentration. The growth control wells contained 0.1 ml of the corresponding diluted inoculum suspension and 0.1 ml of the drug diluent without antifungal agents.

Test procedure

Test was performed in sterile microtiter plates. Aliquots of 100 µl of drug dilutions were dispensed in 1–10 microtiter wells. To each well, 100 µl of inoculum was added. Growth control well was set up with inoculum and without antifungal drug. All microdilution trays were incubated at 28°C without agitation.

Reading of the results

The MIC was taken as the lowest concentration of antifungal agent that substantially inhibits growth of the organism as detected visually. For the conventional microdilution procedure, the growth in each MIC well is compared with that of the growth control with the aid of reading mirror. Each microtiter well was then given a numerical score as follows:

4 - No reduction in growth
3 - Slight reduction in growth or approximately 80% of growth control (drug-free medium)
2 - Prominent reduction in growth or approximately 50% of growth control
1 - Slight growth or approximately 25% of growth control
0 - optically clear or absence of growth
MIC results recorded in µg/ml.

Drug	Endpoint for MIC
Itraconazole (100%)-(80)	Score "0"
Fluconazole, ketoconazole	Score "2" or less

Results

A total of 60 dermatophytes were isolated from the 170 clinically suspected tinea patients. *Trichophyton rubrum, Trichophyton rubrum mentagrophytes,* and *Trichophyton rubrum tonsurans* together constituted

65% of the isolates. The results of antifungal susceptibility test including MIC 50 and MIC 90 for griseofulvin, ketoconazole, fluconazole, itraconazole, and terbinafine, for all the isolates of this study, are mentioned in Tables 1-5, respectively.

Discussion

Although fungi are not known to cause outbreaks, the incidence of severe systemic fungal infections is increasing, mainly because of

Table 1: Susceptibility test results for griseofulvin (range tested 0.03–16 µg/ml)

Species	Drug concentrations (in µg/ml)						
	0.03	0.06	0.12	0.25	≥0.5	MIC 50	MIC 90
T. rubrum (*n*=16)	0	2 (12.5)	6 (37.5)	8 (50)	-	0.12	0.25
T. mentagrophytes (*n*=13)	3 (23)	4 (30.7)	0	6 (46)	-	0.06	0.25
T. tonsurans (*n*=10)	3 (30)	4 (40)	3 (30)	-	-	0.06	0.12
T. verrucosum (*n*=8)	3 (37.5)	2 (25)	0	3 (37.5)	-	0.06	0.25
T. violaceum (*n*=6)	0	3 (50)	3 (50)	-	-	0.06	0.12
T. schoenleinii (*n*=2)	0	1 (50)	0	1 (50)	-	0.06	0.25
E. floccosum (*n*=2)	1 (50)	0	0	1 (50)	-	0.03	0.25
M. gypseum (*n*=2)	0	2 (100)	-	-	-	<0.06	0.06
M. audouinii (*n*=1)	0	0	1 (100)	-	-	<0.12	0.12

T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii, MIC: Minimum inhibitory concentration

Table 2: Susceptibility test results for ketoconazole (range tested 0.03–16 µg/ml)

Species	Drug concentrations (in µg/ml)							
	0.03	0.06	0.12	0.25	0.5	≥1	MIC 50	MIC 90
T. rubrum (*n* = 16)	0	2 (12.5)	7 (43.7)	4 (25)	3 (18.7)	-	0.12	0.5
T. mentagrophytes (*n* = 13)	3 (23)	0	5 (35.7)	5 (35.7)	-	-	0.12	0.25
T. tonsurans (*n* = 10)	0	6 (60)	2 (20)	2 (20)	-	-	0.06	0.25
T. verrucosum (*n* = 8)	0	3 (37.5)	1 (12.5)	0	4 (50)	-	0.12	0.5
T. violaceum (*n* = 6)	2 (33.3)	0	4 (66.6)	-	-	-	0.03	0.12
T. schoenleinii (*n* = 2)	0	0	1 (50)	1 (50)	-	-	0.12	0.25
E. floccosum (*n* = 2)	1 (50)	0	0	0	1 (50)	-	0.03	0.5
M. gypseum (*n* = 2)	0	0	1 (50)	0	1 (50)	-	0.12	0.5
M. audouinii (*n* = 1)	0	0	0	0	1 (100)	-	<0.5	0.5

T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii, MIC: Minimum inhibitory concentration

Table 3: Susceptibility test results for fluconazole (range tested 0.06–32 µg/ml)

Species	Drug concentrations (in µg/ml)								
	≥0.5	1	2	4	8	16	32	MIC 50	MIC 90
T. rubrum (*n*=16)	0	8 (50)	5 (31.2)	3 (18.7)	-	-	-	1	4
T. mentagrophytes (*n*=13)	0	0	7 (53.8)	2 (15.3)	4 (30.7)	-	-	2	8
T. tonsurans (*n*=10)	0	0	5 (50)	3 (30)	2 (20)	-	-	2	8
T. verrucosum (*n*=8)	0	0	4 (50)	4 (50)	-	-	-	2	4
T. violaceum (*n*=6)	0	0	0	5 (83.3)	-	1 (16.6)	-	4	16
T. schoenleinii (*n*=2)	0	0	1 (50)	-	-	1 (50)	-	2	16
E. floccosum (*n*=2)	0	0	0	2 (100)	-	-	-	<4	4
M. gypseum (*n*=2)	0	0	0	1 (50)	-	-	1 (50)	4	32
M. audouinii (*n*=1)	0	0	0	1 (100)	-	-	-	<4	4

T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii, MIC: Minimum inhibitory concentration

Table 4: Susceptibility test results for itraconazole (range tested 0.007–4 μg/ml)

Species	Drug concentrations (in μg/ml)								
	0.0075	0.015	0.03	0.06	0.12	0.25	≥0.5	MIC50	MIC 90
T. rubrum (*n*=16)	0	4 (25)	1 (6.25)	4 (25)	4 (25)	3 (18.7)	-	0.06	0.25
T. mentagrophytes (*n*=13)	2 (15.3)	5 (35.7)	0	2 (15.3)	1 (7.6)	-	-	0.015	0.12
T. tonsurans (*n*=10)	0	1 (10)	4 (40)	3 (30)	0	2 (20)	-	0.03	0.25
T. verrucosum (*n*=8)	0	1 (12.5)	1 (12.5)	4 (50)	0	2 (25)	-	0.06	0.25
T. violaceum (*n*=6)	0	0	5 (62.5)	1 (16.6)	-	-	-	0.03	0.12
T. schoenleinii (*n*=2)	0	0	1 (50)	0	1 (50)	-	-	0.03	0.12
E. floccosum (*n*=2)	0	0	1 (50)	1 (50)	-	-	-	0.03	0.06
M. gypseum (*n*=2)	0	1 (50)	0	1 (50)	-	-	-	0.015	0.06
M. audouinii (*n*=1)	0	0	1 (100)	-	-	-	-	<0.03	0.03

T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii, MIC: Minimum inhibitory concentration

Table 5: Susceptibility test results for terbinafine (range tested 0.007–4 μg/ml)

Species	Drug concentrations (in μg/ml)						
	0.007	0.015	0.03	0.06	≥0.12	MIC 50	MIC 90
T. rubrum (*n*=16)	3 (18.7)	3 (18.7)	5 (31.2)	5 (31.7)	-	0.03	0.06
T. mentagrophytes (*n*=13)	3 (23)	5 (35.7)	3 (23)	2 (15.3)	-	0.015	0.06
T. tonsurans (*n*=10)	0	0	6 (60)	4 (40)	-	0.03	0.06
T. verrucosum (*n*=8)	3 (37.5	1 (12.5)	2 (25)	2 (25)	-	0.015	0.06
T. violaceum (*n*=6)	3 (50)	2 (33.3)	0	1 (16.6)	-	0.0075	0.06
T. schoenleinii (*n*=2)	0	2 (100)	-	-	-	<0.015	0.015
E. floccosum (*n*=2)	1 (50)	0	0	1 (50)		0.0075	0.06
M. gypseum (*n*=2)	0	1 (50)	1 (50)	-	-	0.015	0.03
M. audouinii (*n*=1)	0	0	1 (100)	0	-	<0.03	0.03

T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii, MIC: Minimum inhibitory concentration

the explosive growth in the number of patients with compromised immune system. Opportunistic fungal infections are common among patients who have acquired immunodeficiency syndrome or who have had medical procedures that suppress the immune system such as organ transplantation and chemotherapy. The indiscriminate use of antibiotics also contributes to this issue. Hence, it is necessary to have antifungals available for the efficient control of fungal infections.[6]

A few decades ago, the number of antifungal drugs available was small and fungal infections were easier to treated as they were often limited to superficial mycoses such as athlete's foot, thrush caused by *Candida albicans*, *Cryptococcosis*, ringworms (keratomycoses), and a few cases of deep-seated mycoses.[7] Now, although several antifungal agents are available that are more potent and less toxic and have improved pharmacokinetics, their cellular targets are limited because of the similarity existing between fungi and hosts, both being eukaryotes. The inadequate use or dosage of drugs contributes to the failure in eliminating the disease agent completely, encouraging growth of the most resistant strains. Decrease in drug uptake, structural alterations in the target site, and an increase in drug efflux or in intracellular target levels are important mechanisms of drug resistance among dermatophytes.[6]

Although MIC-based tests to detect drug resistance among dermatophytes are widely used, the MIC value for any drug depends on the quality of the specimen, quantity of the inoculum, composition and pH of the medium, temperature and time of incubation, drug solvent, and growth curve.[8-10] In addition, the conidiation of some dermatophytes is very poor on standard fungal media. The Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi (M38-A)[4] standardized by the CLSI does not explicitly address the antifungal susceptibility of dermatophytes.[11] However, adaptations of the M38-A protocol for susceptibility testing of dermatophytes are proven to have excellent reproducibility of MIC data which are being widely used.[10,11] However, it has also been reported that MICs of antifungals obtained with hyphal fragments inocula from other filamentous fungi were substantially higher than those obtained with conidial inocula.[12]

Table 6 summarizes comparison of dermatophyte susceptibilities to the five antifungals tested with similar study from a different region

Table 6: Comparison of *in vitro* activities of 5 antifungal by microdilution method

Dermatophytes	MIC (µg/ml)	Griseofulvin		Ketoconazole		Fluconazole		Itraconazole		Terbinafine	
		Present study	Ref1	Present study	Ref1	Present study	Ref1	Present study	Ref1	Present study	Ref1
T. rubrum	Range	0.06–0.12	0.16–5.12	0.06–0.5	0.01–3.84	1–4	0.16–20.48	0.015–0.25	0.03–3.84		0.001–0.08
	MIC 50	0.12	1.26	0.12	0.24	1	1.28	0.06	0.24	0.03	0.005
	MIC 90	0.25	2.56	0.5	1.92	4	10.24	0.25	1.92	0.12	0.04
T. mentagrophytes	Range	0.06–0.25	0.32–5.12	0.12–0.25	0.01–0.96	2–8	0.08–20.48	0.007–0.12	0.03–1.92		0.002–0.16
	MIC 50	0.06	1.28	0.12	0.12	2	1.28	0.015	0.24	0.015	0.06
	MIC 90	0.25	2.56	0.25	0.24	8	10.24	0.12	0.96	0.12	0.08
T. tonsurans	Range	0.03–0.12	0.64–5.12	0.06–0.25	0.01–0.48	2–8	0.16–20.48	0.015–0.25	0.48–7.68		0.005–0.04
	MIC 50	0.06	1.26	0.06	0.06	2	2.56	0.03	1.92	0.03	0.01
	MIC 90	0.12	2.56	0.25	0.12	8	5.12	0.12	3.84	0.12	0.02
T. verrucosum	Range	0.03–0.25	0.32–1.26	0.06–0.5	0.03–0.12	2–4	0.32–5.12	0.015–0.25	0.12–0.92		0.02–0.08
	MIC 50	0.03	0.64	0.06	0.03	2	2.56	0.06	0.24	0.015	0.04
	MIC 90	0.25	1.28	0.5	0.12	4	5.12	0.25	0.96	0.06	0.08
T. violeceum	Range	0.06–0.12	0.32–5.12	0.03–0.12	0.03–1.92	4–16	0.16–10.24	0.03–0.06	0.01–0.96		0.001–0.08
	MIC 50	0.06	1.28	0.03	0.48	4	2.56	0.03	0.12	0.007	0.01
	MIC 90	0.12	2.56	0.12	0.96	16	5.12	0.06	0.48	0.06	0.04
T. schoenleinii	Range	0.06–0.25	0.32–2.56	0.12–0.25	0.06–0.96	2–16	0.32–10.24	0.03–0.12	0.12–0.96		0.01–0.08
	MIC 50	0.06	0.64	0.12	0.24	2	2.56	0.03	0.24	<0.015	0.02
	MIC 90	0.25	1.28	0.25	0.48	16	5.12	0.12	0.48	0.015	0.04
M. gypseum	Range	0.06–0.12	0.64–5.12	0.12–0.5	0.01–3.84	4–32	0.16–40.96	0.015–0.06	0.03–0.96		0.005–0.64
	MIC 50	0.06	1.28	0.12	0.96	4	10.24	0.015	0.12	0.15	0.08
	MIC 90	0.12	2.56	0.5	1.92	32	20.28	0.06	0.48	0.25	0.32
M. audouinii	Range	0.12	0.32–5.12	0.5	0.03–1.92	4	0.32–10.24	0.13	0.03–0.96		0.005–0.16
	MIC 50	<0.12	1.28	<0.5	0.12	<4	2.56	<0.03	0.12	<0.12	0.02
	MIC 90	0.12	2.56	0.5	0.96	4	5.12	0.03	0.48	0.12	0.08

T. rubrum: Trichophyton rubrum, T. mentagrophytes: Trichophyton mentagrophytes, T. tonsurans: Trichophyton tonsurans, T. verrucosum: Trichophyton verrucosum, T. violaceum: Tricholosporum violaceum, T. schoenleinii: Trichophyton schoenleinii, E. floccosum: Epidermophyton floccosum, M. gypseum: Microsporum gypseum, M. audouinii: Microsporum audouinii, MIC: Minimum inhibitory concentration

of South India in 2013. The MIC values of all dermatophytes were lower for griseofulvin in the present study compared to the study by Indira G; on the other hand, MICs for ketoconazole are higher in the present study.

The present study and the study conducted by Ghannoum *et al.*, in 2004, show higher MIC values of griseofulvin and fluconazole in the previous study. Similarities were seen in the MIC values for itraconazole and terbinafine.[11] In a multicenter study performed by Espinel–Ingroff *et al.* found the lowest intra- and inter-laboratory agreement for itraconazole (59–79% and 59–91%). All the above results of this present study almost correlate with the previous studies conducted by Fernandez Torres *et al.*[13] 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 among the laboratories.

Conclusion

As we can see by comparing the data of this study with other previous studies, antifungal susceptibility patterns of dermatophytes vary in different geographical areas and populations and also change with time. MICs of some dermatophyte antifungal combinations are found to be high and are likely to lead to treatment failure; hence, antifungal susceptibility testing for dermatophytes should be strongly considered, especially in non-responding cases.

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