ORIGINAL ARTICLE

In-Vitro Susceptibility Testing of Dermatophytes Isolated in Delhi (India) Against Five Antifungal Drugs

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ABSTRACT

Background: Over the past few decades, cases of dermatophytosis have been on the rise. Recently, the introduction of newer, less toxic antifungal drugs has improved treatment options. However, the prolonged use of antifungals has led to the emergence of acquired resistance among strains that were previously susceptible, as well as an increase in infections caused by less common species. This scenario underscores the growing need for rapid and accurate antifungal susceptibility testing methods. In this study, antifungal susceptibility was assessed using the in-vitro micro broth dilution method, adhering to the CLSI M38-A guidelines

Methods: 60 clinical specimens were collected from Nail and skin of the patients of dermatophytosis from Delhi (India). Minimal inhibitory concentration (MIC) was performed in microtiter plates with U-bottom and incubated at 35° C. Reading were taken after 48 & 96 hrs of incubation for Trichophyton mentagrophytes and Trichophyton rubrum, against 5 antifungal drugs namely fluconazole, itraconazole (triazoles), griseofulvin, terbinafine and Luliconazole.

Results: Most of the dermatophytes had uniform patterns of susceptibility to the antifungal agents tested. Low MIC values as 0.03µg/mL were found for 33.3%, 31.6% and 15% of isolates for itraconazole and terbinafine, respectively.

Conclusion: In conclusion, it may be useful to undertake periodical screening programs to detect the antifungal susceptibility of newer antifungal agents.

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Introduction

Dermatophytes, a group of keratinophilic filamentous fungi thriving on the keratin substrate are the etiological agents responsible for causing Dermatophytosis in human and animal worldwide. Dermatophytosis is a fungal infection of the skin, hair and nail caused as a result on colonization of the keratinized layers of the body by organisms belonging to the three genera namely Trichophyton, Microsporum and Epidermophyton. More predominant in the tropical and subtropical countries; especially in the developing countries like India where the hot climate and humid weather is favorable to the acquisition and maintenance of the disease.[1] The World Health Organization estimates global prevalence of Dermatophytosis to be approximately 20percent. The incidence of onychomycosis has been reported to be 0.5-5% in general population and it may be as high as 45 percent as detected in a five year study from North India. The reported prevalence of onychomycosis varies from 2% to 8% [2] worldwide and from 0.5% to 5% in India.[3]

Oral antifungal therapy such as triazoles (itraconazole, fluconazole), allylamine (terbinafine), griseofulvin and Luliconazole are current systemic treatment of choice for Dermatophytosis that do not respond to the tropical therapy.[4] Role of antifungal in reducing the fungal load to have the association of immune clearance the degree of clinically failure is 25-40%.[5] However, the relapse rate is upto 10 percent in toenail onychomycosis after cessation of therapy. The variable activity of these drugs leading to treatment failure can be attributed to poor patient compliance, infection with the new strain, lack of drug penetration into the nail, medication bioavailability, or drug interactions and resistance.[6-8] In-vitro antifungal susceptibility tests could help in optimizing the therapy and to select an effective antifungal agent for dermatophytosis.[9-10]

Therefore, the purpose of this study was to detect the in-vitro antifungal susceptibility testing of dermatophytes isolated from superficial skin and nail infections against antifungal agents like fluconazole, itraconazole, terbinafine, griseofulvin and luliconazole at a tertiary care hospital of North India using the broth micro dilution method (M38-A) according to CLSI standards (previously the NCCLS method).

Materials and Methods

Study group

The present study was conducted on 50 clinically diagnosed patients with dermatophytosis of skin and nail attending dermatology out patient centre of a tertiary care hospital, Delhi. The data from the patients were 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 by scraping such as skin, nail collected in the Mycology section of the department of Microbiology, GTB Hospitral, Delhi. Each clinical sample was mixed with a drop of 10% potassium hydroxide (KOH) and examined under a light microscope. Part of the specimen was then cultured on Sabouraud's dextrose agar (Hi-media) containing chloramphenicol (0.05 g/l), gentamicin (20 mg/l), and cycloheximide (0.5 g/l). The inoculated tubes were kept at 25°C to promote optimal fungal growth. Upon observing growth,

the causative organism was identified based on the unique morphology of the colony and the microscopic characteristics of the fungus observed on a Lacto Phenol Cotton Blue (LPCB) slide. These isolates were further analyzed for antifungal sensitivity.

Preparation Antifungal agents as per CLSI M-38 A -:

Antifungal drugs: Antifungal drugs fluconazole, itraconazole, terbinafine & griseofulvin, Luliconazole were obtained from Sigma Pvt. Ltd. Fluconazole was dissolved in sterile distilled water and all other drugs were dissolved in 100% Dimethyl Sulfoxide (Invitrogen) following the protocol of CLSI M – 38 A. Stock solutions of 1,000 µg/ ml were prepared for each drug and stored at -20°C till tests were performed. All the drugs were further diluted to two fold dilutions were performed. All working solution of drugs diluted in DMSO should always be prepared in tubes with the same solvent (DMSO) before transferring onto plates, whereas serial dilutions of water soluble drugs are prepared directly in Microtitre plate. The final concentrations ranged from 0.125 to 64 µg/mL for fluconazole, 0.03 to 16 µg/mL for,itraconazole and terbinafine, and 0.03 to 8 µg/mL for griseofulvin, Luliconzole 0.00625 to2 µg/mL.

Preparation of inoculum:- The MIC was performed according to CSLI (M38-A) modified method in a polysterene microtiter plates with U-bottom wells. Dermatophyte inoculum suspensions were prepared using seven-day-old sub-cultures grown on Sabouraud dextrose agar at 25°C. About 10 mL of normal saline was poured over the fungal colonies, and the surface was gently scraped with the tip of a sterile loop to produce the suspensions. After collecting the mixture of conidia and hyphal fragments, it was transferred into sterile tubes and allowed to settle for 15 to 20 minutes at room temperature. The optical density of these suspensions was measured at 530 nm, adjusted to a transmittance of 65 to 70% (equivalent to 1 to 4 X 10^6 cells/mL), and then diluted with RPMI 1640 medium and MOPS (4-Morpholinepropanesulfonic acid) from Sigma Chemical Co., St. Louis, Mo. A 1:50 dilution was made to achieve a final inoculum concentration of approximately 0.4 X 10^3 to 5 X 10^4 cells/mL. The density of the inoculum was confirmed through quantitative colony counts using a Colony Counter unit.

Test procedure (Susceptibility testing):- 100 µL RPMI & antifungal was distributed in all wells and 200 µL of RPMI was taken as growth control in the u-bottom microtitre plate . 100 µL of inoculum was added in each well except growth control well.Growth and sterility control wells also maintained for each assay and all the tests were performed in duplicate. MICs of quality-control ATCC strains of C. parapsilosis ATCC-22019 and C. krusei ATCC-6258 were also used. Plates were incubated for 96 hrs at 350C.MICs were based on the lowest drug dilution that inhibited at least 80% of growth compared with the control. Dermatophytes, MIC measurements were taken using a viewing mirror.

Endpoint determination

Visual assessments for endpoint determination were conducted every 24 hours for up to 96 hours, observing the growth in a control well without the drug. Minimum Inhibitory Concentrations (MICs) were evaluated in duplicate across three separate tests. The MIC was identified as the least concentration of the drug that prevented fungal growth. For azole medications, the MIC was

recognized as the concentration that achieved an 80% reduction in growth relative to the control. Growth sterility controls were included in each test. For Terbinafine, the MIC was the concentration that completely inhibited growth by 100%, whereas for Fluconazole, it was 50%. For Itraconazole and Griseofulvin, the MIC was determined as the concentration that visibly inhibited approximately 80% of fungal growth. MIC50 was determined by the concentration at which 50% of the isolates were inhibited, and MIC90 was noted at the concentration inhibiting 90% of the isolates. The MIC values were determined based on the extent of growth inhibition. Luliconazole MICs were defined according to the established MIC reports.

Statistical analysis:- MIC data were transformed to a normal distribution using the ANOVA was used to compare each antifungal agent vs. its MIC for each isolate . Differences in MIC values were analyzed using the Tukey test (a = 0.05). Mean geometric MIC values were determined for all the isolates tested, and the MIC values at which 50% and 90% of the isolates were inhibited (MIC50 and MIC90, respectively) were determined only for groups containing ‡T.R 15 & T.M 35 isolates. SAS using software (Version 6.12; SAS Institute, NC State University, Raleigh, NC, USA) . $P < 0.05$ was considered not significant.

Results

A total of 50 dermatophytes strains, including Trichophyton rubrum (n = 15), T. mentagrophytes (n = 35) from nail (35) and skin(15) were tested.

Table 1 Pattern of in-vitro activity of 5 antifungal against 50 clinical isolates of *T. rubrumand T. mentagrophytes* from dermatophytosis patients by micro dilution testing

SAMPLE S.no.	Samples	Species	Fluconazole Range (ug/ml)	Itraconazole Range (ug/ml)	Terbinafine Range (ug/ml)	Griseofulvin Range (ug/ml)	Luliconazole Range (ug/ml)
1	Nail	T.M	1	0.0313	0.125	0.0313	0.00005
2	Nail	T.R *	64	16	16	0.5	0.00005
3	Nail	T.M	64	0.625	0.25	0.125	0.00005
4	Nail	T.R *	1	0.5	0.625	0.625	0.00005
5	Nail	T.M	2	4	0.125	8	0.00005
6	Nail	T.R *	4	8	8	0.0313	0.00005
7	Nail	T.M	4	8	0.125	4	0.00005
8	Nail	T.M	8	0.5	0.125	0.25	0.00005
9	Nail	T.M	8	0.0313	0.5	0.5	0.00025
10	Nail	T.M	8	16	0.0313	0.5	0.00005
11	Nail	T.M	4	0.125	0.0625	0.125	0.00005
12	Nail	T.M	2	0.125	0.0313	0.125	0.00005
13	Nail	T.R *	64	4	0.125	0.625	0.00025

14	Nail	T.R *	1	8	0.625	0.0313	0.00005
15	Nail	T.M	64	0.5	8	16	0.00025
16	Skin	T.R *	128	0.0313	0.0313	0.0313	0.00005
17	Skin	T.M	8	0.5	0.125	0.25	0.00005
18	Skin	T.M	32	0.25	0.125	0.0313	0.00005
19	Skin	T.M	8	2	0.5	0.125	0.00005
20	Skin	T.R *	4	2	0.0313	4	0.00025
21	Skin	T.R *	2	0.0313	0.0313	0.125	0.00005
22	Skin	T.M	1	0.0313	0.0313	0.125	0.00005
23	Skin	T.M	32	0.0625	0.625	8	0.00025
24	Skin	T.M	1	0.0313	0.5	0.625	0.00005
25	Skin	T.M	0.5	0.625	0.0313	0.625	0.00005
26	Skin	T.M	0.5	0.125	0.0313	0.0313	0.00005
27	Skin	T.R *	0.5	4	0.125	0.0313	0.00005
28	Skin	T.R *	64	2	8	16	0.00025
29	Skin	T.R *	128	0.0313	0.125	0.125	0.00005
30	Skin	T.M	8	0.313	0.625	0.25	0.00005
31	Skin	T.M	1	0.0313	0.125	0.625	0.0012
32	Skin	T.M	1	0.5	0.125	0.125	0.00005
33	Skin	T.M	0.5	2	0.125	0.125	0.00005
34	Skin	T.R *	0.5	0.625	0.0313	0.5	0.0012
35	Skin	T.R *	1	0.125	0.0313	0.625	0.00005
36	Skin	T.M	1	0.125	0.125	0.125	0.00005
37	Skin	T.R*	2	0.125	0.0313	0.125	0.00005
38	Skin	T.M	1	0.0313	0.0313	0.5	0.0012
39	Skin	T.M	2	0.0313	0.0125	0.125	0.00005
40	Skin	T.M	1	0.0313	0.0313	0.0313	0.00005
41	Skin	T.R *	64	0.625	0.0313	0.125	0.00025
42	Skin	T.M	1	0.625	0.0625	0.0313	0.00005
43	Skin	T.M	1	0.0313	0.0313	0.0313	0.00005
44	Skin	T.M	2	0.0313	0.625	0.0625	0.00005
45	Skin	T.M	1	0.625	0.125	0.0313	0.00025
46	Skin	T.M	2	0.125	0.0313	0.625	0.00005
47	Skin	T.M	1	0.125	0.625	0.0313	0.00005
48	Skin	T.M	2	0.0313	0.625	0.0313	0.00025
49	Skin	T.M	1	0.0313	0.0313	0.0625	0.00005
50	Skin	T.M	2	0.0125	0.0313	0.0313	0.00005

Summarizes the MIC ranges, concentrations inhibiting 50% MIC50) and 90% (MIC90) of the isolates and Nail (15)& Skin(35) in T.R & T.M of the MICs against the five antifungal drugs against 50 strains of dermatophytes. The MIC ranges of Fluconazole, Itraconazole (triazoles), Terbinafine, Griseofulvin and Luliconazole for within the values standardized by CLSI document M-38-A.

Table 2 Activity of fluconazole, Itraconazole, Terbinafine,Griseofulvin and Luliconazole against dermatophytes by Minimum inhibitory concentrations (MICs) for tested antifungal drugs against a range

Antifungal drugs	**Break points**	**Species (no.)**	**R**	**SDD**	**S**	**MIC 50**	**MIC 90**	**GM**
Fluconazole	S≤ 8, SDD ≤ ≥64 R≥64	T.R 15 T.M 35	4 4	0 2	11 29	8	32	16.09
Itraconazole	S≤0.125, SDD ≤ ≥0.25-0.5 R≥1	T.R 15 T.M 35	7 5	2 3	6 27	0.125	1	1.68
Terbinafine	S≤0.125, SDD ≤ ≥0.25-0.5 R≥1	T.R 15 T.M 35	2 2	2 2	11 31	0.5	2	0.97
Griseofulvin	S≤0.125, SDD ≤ ≥0.25-0.5 R≥1	T.R 15 T.M 35	2 4	2 6	11 25	0.5	2	1.32
Luliconazole	S≤0.0005, SDD ≤ ≥0.0625-0.0125 R≥0.25	T.R 15 T.M 35	0 0	1 2	14 33	0.0005	0.016	0.00015

Antifungal activity against dermatophyte species

The he dermatophytes most frequently isolated were T. rubrum and T. mentagrophytes, as indicated in Table 1. Terbinafine demonstrated superior antifungal efficacy against these fungi, with geometric mean (GM) values of 0.014 for T. rubrum and 0.190 for T. mentagrophytes, as shown in Tables 2 and 3. Significant variations were observed in the MIC values of all antifungals tested against the dermatophytes on days 2 and 6, whereas the differences in MIC values between days 2 and 4 were not statistically significant ($P > 0.05$)

Table 3 Species V/S Drugs

Antifungal drugs	Break points	Species (no.)	R	SDD	S	MIC 50	MIC 90	GM
Fluconazole	S≤ 8, SDD ≤ ≥64 R≥64	T.R 15 T.M 35	4 4	0 2	11 29	8	32	16.09
Itraconazole	S≤0.125, SDD ≤ ≥0.25-0.5 R≥1	T.R 15 T.M 35	7 5	2 3	6 27	0.125	1	1.68
Terbinafine	S≤0.125, SDD ≤ ≥0.25-0.5 R≥1	T.R 15 T.M 35	2 2	2 2	11 31	0.5	2	0.97
Griseofulvin	S≤0.125, SDD ≤ ≥0.25-0.5 R≥1	T.R 15 T.M 35	2 4	2 6	11 25	0.5	2	1.32
Luliconazole	S≤0.0005, SDD ≤ ≥0.0625-0.0125 R≥0.25	T.R 15 T.M 35	0 0	1 2	14 33	0.0005	0.016	0.00015

Minimum inhibitory concentrations (MICs) for tested antifungal drugs against a range of organisms.

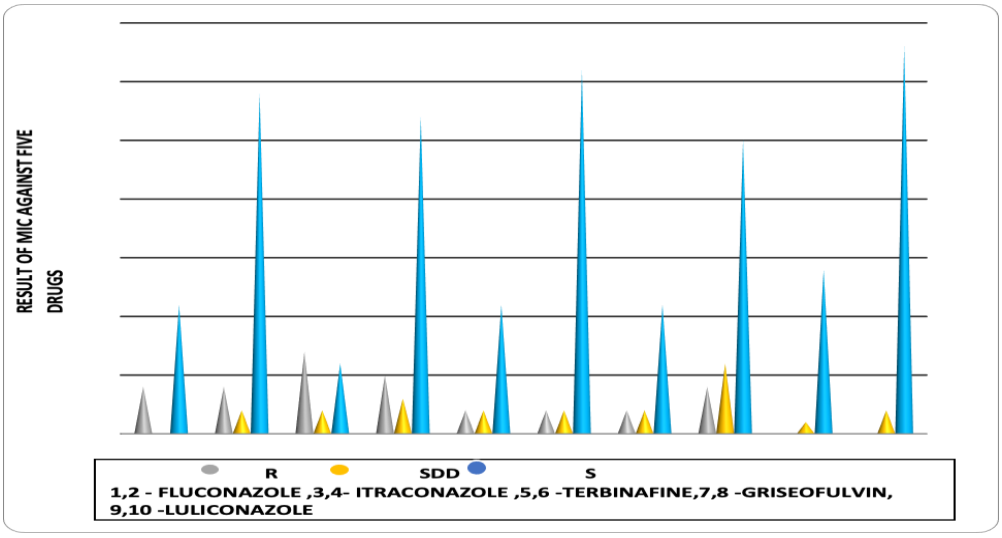

Figure 1 Bar chart for species v/s drugs

In case of fluconazole total 16 % cases resistant(R), 4 % cases susceptible but dose depended (SDD) & 80% cases are sensitive(S).Itraconazole- 24 % cases R,12% cases SDD & 64% S, Terbinafine – 8 % cases are R, 6 % SDD & 86 % cases are Sensitive . Griseofulvin - 12% R, 16% SDD & 72 % sensitive. Luliconazole – 6 % SDD & 94 % are sensitive.

Table 4 Total percentages of Resistant, SDD & Sensitive

Antifungal drugs	**Resistant % N = 50**	**Susceptible but dose depended (SDD)% N = 50**	**Sensitive % N = 50**
Fluconazole	16%	4%	80%
Itraconazole	24%	12%	64%
Terbinafine	8%	6%	86%
Griseofulvin	12%	16%	72%
Luliconazole	0%	6%	94%

Table 5 Percentage of resistant isolates

Antifungal drugs	**Species**	**Resistant % N = 50**	**Susceptible but dose depended (SDD)% N = 50**	**Sensitive % N = 50**
Fluconazole	T.R T.M	3.75%	4%	80%
Itraconazole	T.R T.M	24%	12%	64%
Terbinafine	T.R T.M	8%	6%	86%
Griseofulvin	T.R T.M	12%	16%	72%
Luliconazole	T.R T.M	0%	6%	94%

Note: I – Itraconazole, T –Terbinafine, G-Griseofulvin, L -Luliconazole

Discussion

Treating fungal infections is more challenging than treating bacterial infections due to the eukaryotic nature of fungal cells, which are structurally closer to human cells than bacteria. This similarity means that many antifungal drugs can also be toxic to humans.[11-12] Additionally, fungal cells possess a detoxification mechanism that can alter many antibiotics, often through hydroxylation.[13] Effective antifungal agents often work by removing sterols from the membrane or inhibiting their synthesis. Most target the production or function of ergosterol, a crucial element of the fungal cell membrane.[14-20]

Currently, there are no universally accepted thresholds (epidemiological cutoff values) to define susceptibility or resistance of dermatophyte strains to antifungals across various regions. [21-25] Therefore, our minimum inhibitory concentration (MIC) benchmarks for fluconazole, itraconazole, and griseofulvin are based on standardized reference methods and CLSI guidelines.[19] In our study, most antifungal drugs except fluconazole exhibited effective activity against dermatophytes, with itraconazole and terbinafine showing particularly low MIC values and geometric means, corroborating findings by other researchers who noted similar efficacy. These low MICs contribute to the promising treatment outcomes observed for dermatophytosis with these drugs.

Despite fluconazole displaying the highest MIC values among the tested antifungals, we found that T. rubrum strains, which often cause chronic, stubborn infections, were more responsive to this drug compared to T. mentagrophytes and M. canis strains, with geometric means of 7.60 for T. rubrum, 9.96 for M. canis, and 11.31 for T. mentagrophytes.[26-30] This observation aligns with findings by Fernández-Torres et al., who noted fluconazole's higher efficacy against T. rubrum than T. mentagrophytes.

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. But still the efficacy of Terbinafine 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.

Conflict of Interest

The authors declare no potential conflicts of interest or competing interests. The authors received non-financial assistance or grants from public, private, or non-profit funding agencies.

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