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INCREASED RESISTANCE TO ANTI-FUNGAL AGENTS BY DERMATOPHYTES ISOLATED FROM SIKKIM, A HIMALAYAN STATE OF INDIA

Microbiology

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ABSTRACT

Background : Dermatophytic infections and its changing pattern among people of Sikkim over the past few years and the increasing treatment failure with various antifungal agents has brought a need to start study on antifungal susceptibility testing of dermatophytes isolated from this part of the country.

Aims and objectives: To perform antifungal susceptibility testing of the isolated dermatophytes and to determine the minimum inhibitory concentration (MICs) of antifungal agents.

Materials and methods: The study was carried out from May 2016 to October 2016. A total of 60 dermatophytes isolated from the clinical specimens were tested for antifungal susceptibility.

Results: For the isolated dermatophytes, griseofulvin had the lowest MIC, followed by itraconazole and terbinafine. All the isolates showed very high MIC against fluconazole.

Conclusion: The present study highlights the drug sensitivity pattern of dermatophytes in this part of the country. Dermatophytosis is caused by multiple species, which cannot be pin point on the clinical ground and different species show different drug susceptibility pattern, which thereby suggest multidrug therapy could be a better option for the management of a case.

KEYWORDS

Antifungal agents, Antifungal susceptibility test, Dermatophytes, Minimum inhibitory concentration, Sikkim.

INTRODUCTION:

Dermatophytosis is one of the most common diseases in human beings commonly referred as "ringworm," a superficial infection of keratinized tissue caused by organisms of three genera of closely related fungi known as dermatophytes.[1] Dermatophytes are classified into *Epidermophyton*, *Microsporum* and *Trichophyton*. Dermatophytes are keratinophilic fungi which are capable of invading the keratinous tissue of living animals.[2, 3] Though it does not cause mortality, but it causes high morbidity and worsens the quality of patients' life. Today, we are facing an onslaught of chronic and recurrent dermatophytosis in volumes never encountered previously.[4] Besides the availability of wide range of antifungal agents, the reason for failure of treatment possibly due to resistance to the agent by dermatophyte implicated in mycoses has been suggested by some authors.[5] The exact role of drug resistance in treatment failure is not clearly understood. The reason for the increasing drug resistance could be due to over the counter availability of the drugs, misuse of drugs (under dose, overuse, and incomplete duration of treatment).

All the species of dermatophytes do not have the same pattern of susceptibility to different antifungal agents. In vitro, antifungal susceptibility testing could therefore, prove helpful in the better management of the dermatophytosis because effective antifungal agents for the optimization of antifungal therapy can be selected by determining minimum inhibitory concentration (MICs) of the agents.[6] Broth macro and micro dilution methods, agar dilution and disc diffusion methods are routinely used for this purpose.[7, 8] For determining MICs, Clinical and Laboratory Standards Institute (CLSI) approved protocol M38-A2 for filamentous fungi including dermatophytes has been recommended in its guidelines of 2008.[9] Involvement of dermatophytes in the causation of dermatophytosis differ from place to place. Different species of dermatophytes show different anti-fungal susceptibility pattern. It is not feasible to determine MIC of anti-fungal agents for every dermatophyte isolated from individual patient. Therefore, Mycologist should be encouraged to perform anti-fungal susceptibility of dermatophytes isolated from their areas and communicate the same to the dermatologist for proper management of dermatophytosis. They should generate and maintain data on anti-fungal susceptibility of dermatophytes isolated from any given area on at least yearly basis.

Although a number of studies on clinico-mycological aspects of dermatophytosis have been reported from different parts of India but, no report on this aspect and the antifungal susceptibility testing of dermatophytes from Sikkim is available. The present study aims to evaluate the antifungal susceptibility pattern of the isolated dermatophytes.

MATERIALS AND METHODS:

This study was conducted in the department of Microbiology and Dermatology, Sikkim Manipal Institute of Medical Sciences (SMIMS), Tadong.

Clinical samples from the suspected patients (192; 156 skin scrapings, 30 nail clips and 06 plucked hair strands) were collected and the dermatophytes were isolated from the sample. Total of 60 dermatophytes isolated from the clinical specimens (58 *Trichophyton* species and 2 *Epidermophyton* species) were subjected for the further study.[3]

The broth microdilution assay for antifungal susceptibility testing of dermatophytes according to CLSI guidelines in the document M38-A2 (2008) was performed for 60 isolated dermatophytes. The four most commonly used antifungal drugs in powdered form were used in the study: itraconazole (17000000 European pharmaceutical, Sigma-Aldrich), fluconazole (PHR 1160-1G, Sigma-Aldrich), terbinafine hydrochloride (PHR 1160-1G, Sigma-Aldrich) and griseofulvin (PHR 1534-1G, Sigma-Aldrich).

Drug dilution:

The stock dilutions of the above drugs were prepared in Dimethyl Sulfoxide (DMSO, HiMedia).[6, 9, 10] Antifungal stock solutions were prepared at concentrations hundred times more than the highest concentration tested. Itraconazole: 0.03-16 µg/ml; 16000µg of itraconazole was dissolved in 10 ml DMSO. Fluconazole: 0.125-64 µg/ml; 64000µg of fluconazole was dissolved in 10 ml DMSO. Terbinafine: 0.03-16 µg/ml; 16000µg of terbinafine was dissolved in 10 ml DMSO. Griseofulvin: 0.03-8 µg/ml; 8000 µg of griseofulvin was dissolved in 10 ml DMSO. The prepared stock solution was stored at -70ºC until use. The two-fold dilutions from the stock solution were further prepared in RPMI 1640 medium with L-glutamine and without sodium bicarbonate (HiMedia). These dilutions were used in the test at a pH of 7.0 ± 0.1 with 3-(N-morpholino) propanesulfonic buffer (HiMedia) along with 1N NaOH.[9,10]

Preparation of inoculums of dermatophyte species: Cultures of

dermatophyte species (10-15 days old) grown on Potato Dextrose Agar (PDA) slants at 25ºC were used for inoculum preparation. The fungal growth was covered with 5 ml of sterile normal saline and suspensions prepared by scraping the growth from the surface of the slants with a sterile swab. The suspension contained conidia and hyphal fragments. The heavy particles were allowed to settle down for 10 to 15 minutes. The upper clear suspension was transferred to fresh tube, and its optical density was set equal to 0.5 McFarland standards.[11,12]

Quality control reference strains: *Candida parasilopsis* strain ATCC-22019 was taken as quality control reference strains and their susceptibilities to itraconazole, fluconazole, terbinafine and griseofulvin were also tested.

Test procedure: Flat bottomed, 96 well microtitre plates (coster-3596) having 8 rows and 12 columns were used to perform the susceptibility test. 100µl of inoculum was placed in the wells till 11th well of four columns. The dilutions (100µl) of drugs were added in each well of the plate from left to right. The concentration of drug was highest in the first column and decreased from left to right. The 12th well contained the uninoculated drug control. On fifth column positive growth control without drug was taken. The contents were incubated at 35ºC for 72 hours.[9]

Reading result: The Minimum inhibitory concentration (MIC) is the lowest concentration of an antifungal that substantially inhibits growth of the organism as detected visually. For dermatophytes, MIC is the first well where turbidity is reduced to at least 80% (100% for terbinafine and griseofulvin). These values for each drug were recorded. The growth in each well was compared with that of the growth control with the aid of a reading mirror. MIC ranges, Minimum concentration that inhibited 50% of the isolates (MIC_{50}) and 90% of the isolates (MIC_{90}) and the mean MIC values of each antifungal drug was calculated.[6,10]

Interpretation of result: Interpretive breakpoints have not been established for dermatophytes so the susceptibility or resistance pattern of any antifungal drugs cannot be distinguished till date. The MIC value of each drugs were compared with the finding of different studies.[6,10]

Statistical analysis: The obtained final result of the study was analysed statistically by calculating Chi-square (χ^2) test and the association was studied between different variables. In the Fisher's χ^2 table, the calculated χ^2 value was compared with the highest obtainable by chance at the desired degrees of freedom given in the table under different probabilities such as 0.05, 0.02, 0.01, etc.[13]

RESULTS:

A total of 60 (*Trichophyton mentagrophyte 24, Trichophyton schoenleinii* 20, *Trichophyton tonsurans* 10, *Trichophyton rubrum* 04 and *Epidermophyton floccosum* 02) species of dermatophytes were subjected for antifungal susceptibility testing. In vitro susceptibility showed that griseofulvin had the lowest MIC (<1µg/ml in 56.5%), followed by itraconazole (<1µg/ml in 36.5%) and terbinafine (<1µg/ml in 23.3%). All the isolates showed very high MIC against fluconazole (>8µg/ml in 100%).

The MIC ranges, MIC_{50}, MIC_{90} and the mean MIC values of the each antifungal drug are shown in Table 1. MIC of antifungals for the isolated dermatophytes altogether is shown in Table 2.

Table: 1 MIC values of antifungal drugs against the isolated dermatophytes

Species (no. of isolates)	Antifungal agents	MIC (µg/ml)			
		Range	MIC50	MIC90	Mean
T. mentagrophyte (24)	Itraconazole	0.25-8	4	8	4.89
	Fluconazole	16-64	32	64	46.66
	Terbinafine	0.03125-4	0.5	4	1.197
	Griseofulvin	0.125-4	0.25	4	1.260
T. schoenleinii (20)	Itraconazole	0.125-4	0.5	2	1.0375
	Fluconazole	8-64	16	64	27.2
	Terbinafine	2-8	4	8	5
	Griseofulvin	0.0625-4	0.5	4	1.393
T. tonsurans (10)	Itraconazole	1-8	4	8	3.8
	Fluconazole	32-64	32	64	38.4
	Terbinafine	0.5-8	1	8	2.5
	Griseofulvin	0.25-2	1	2	1.25
T. rubrum (04)	Itraconazole	0.0625-0.125	0.0625	0.125	0.093
	Fluconazole	16-64	16	64	40
	Terbinafine	1-4	1	4	2.5
	Griseofulvin	0.03125-0.0625	0.03125	0.0625	0.497
E. floccosum (02)	Itraconazole	0.25	0.25	0.25	0.25
	Fluconazole	8	8	8	8
	Terbinafine	16	16	16	16
	Griseofulvin	0.03125	0.03125	0.03125	0.03125

Table 2: MIC of antifungals for the isolated dermatophytes

Drugs	Conc. (µg/ml) (%)												Total
	0.03125	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	
Itraconazole	0	2 (3.3)	4 (6.6)	6 (10)	10 (16.6)	6 (10)	6 (10)	14 (23.3)	12 (20)	0			60
Fluconazole			0	0	0	0	0	0	6 (10)	14 (23.3)	20 (33)	20 (33)	60
Terbinafine	2 (3.3)	0	6 (10)	0	6 (10)	8 (13.3)	12 (20)	14 (23.3)	10 (16.6)	2 (3.3)			60
Griseofulvin	4 (6.6)	4 (6.6)	12 (20)	8 (13.3)	6 (10)	8 (13.3)	10 (16.6)	8 (13.3)	0				60

The MIC of itraconazole <1µg/ml was seen in 36.5% and ≥1µg/ml was seen in 63.3% For fluconazole all the isolated showed MIC >8µg/ml. For terbinafine the MIC <1µ/ml was seen in 23.3% and ≥1µg/ml was seen in 76.5%. The MIC of griseofulvin <1µg/ml was seen in 56.5% and ≥1µg/ml was seen in 43.2%.

The MIC of the tested antifungals with the different species of dermatophytes is shown in Table 3. Each of the tested antifungals has shown different concentration as MIC for the various species of dermatophytes. As it is seen that the MIC of griseofulvin is <0.0625 for *T. rubrum* and *E. floccosum* and is >0.125 for *T. mentagrophyte, T. tonsurans* and *T. schoenleinii.*

Table 3: MIC of antifungals in various dermatophytes

Species	Conc. of antifungals (µg/ml)												
T. mentagrophyte (n=24)		64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125
	Itra	-	-	0	10	08	02	0	02	02	0	0	0
	Fluc	12	10	02	0	0	0	0	0	0	0	-	-
	Terbi	-	-	0	0	06	04	02	04	0	06	0	02
	Gris	-	-	-	0	04	06	0	0	04	10	0	0
T. schoenleinii (n=20)	Itra	-	-	0	0	02	02	04	08	02	02	0	0
	Fluc	04	04	08	04	0	0	0	0	0	0	-	-
	Terbi	-	-	0	08	06	06	0	0	0	0	0	0
	Gris	-	-	-	0	04	0	04	06	02	02	02	0
T. tonsurans (n=10)	Itra	-	-	0	02	04	02	02	0	0	0	0	0
	Fluc	02	06	02	0	0	0	0	0	0	0	-	-
	Terbi	-	-	0	02	0	02	04	02	0	0	0	0
	Gris	-	-	-	0	0	04	04	0	02	0	0	0
T. rubrum (n=4)	Itra	-	-	0	0	0	0	0	0	0	02	02	0
	Fluc	02	0	02	0	0	0	0	0	0	0	-	-

	Terbi	-	-	0	0	02	0	02	0	0	0	0	0
	Gris	-	-	-	0	0	0	0	0	0	0	02	02
***E. floccosum* (n=2)**	Itra	-	-	0	0	0	0	0	0	02	0	0	0
	Fluc	0	0	0	02	0	0	0	0	0	0	-	-
	Terbi	-	-	02	0	0	0	0	0	0	0	0	0
	Gris	-	-	-	0	0	0	0	0	0	0	0	02

Itra- Itraconazole (0.03125-16µg/ml)
Fluc- Fluconazole (0.125-64 µg/ml)
Terbi- Terbinafine (0.03125-16µg/ml)
Gris- Griseofulvin (0.03125-8µg/ml)

DISCUSSION AND CONCLUSION:

Dermatophytosis is a fungal infection commonly occurring in tropical countries, often representing a public health problem. The distribution and frequency of dermatophytosis and their etiologic agents vary according to the geographic region, the socio-economic status of the population, the time of study, the climatic variations, the presence of domestic animals, and age.[14,15] A number of antifungal agents have been introduced for treating this condition and more are underway.[16] Different dermatophyte strains have different antifungal susceptibility patterns. Strains of dermatophyte resistant to particular antifungal agent have been reported. [17]

The wide range of new antifungal agents that have been introduced and simultaneously the detection of isolate showing resistance to antifungal agents such as terbinafine, azole group etc., and makes testing of the susceptibility of dermatophytes to these agents more important particularly for surveillance of resistant strains in epidemiological studies. It plays an important role in detecting resistant strains that might help clinicians for better management of the disease caused by them by selecting appropriate therapeutic options for checking further spread.[6] The reason for increasing drug resistance could be due to over the counter availability of the drugs and misuse of drugs.

The determination of in-vitro susceptibility may prove helpful to predict the ability of a given antifungal agent to eradicate dermatophyte. Although a standard reference method for dermatophyte, was not available earlier, a good correlation between the in vitro data, using broth micro dilution method, and clinical outcome has been demonstrated.[18]

Prior to CLSI guidelines of 2008, due to the lack of suitable and effective methods of determining the *in vitro* antifungal susceptibility and the MICs of the antifungal drugs against dermatophytes, it is not possible to ensure effective treatment. A number of techniques have been used for this purpose, e.g., disk diffusion method, broth macro and microdilution method, colorimetric microdilution method, E-test etc. [19-22] Some researchers followed the protocol M38-A of CLSI 2002 for determining the susceptibility of dermatophytes that was intended for filamentous fungi. [23] Later, the document was modified to M38-A2 by CLSI in 2008. This document also includes the protocol for dermatophytes which has been followed by us for determining the MIC values of itraconazole, fluconazole, terbinafine and griseofulvin against different dermatophyte species. In the present study, incubation was done for all the species of dermatophytes at 35°C as mentioned in the M38-A2 protocol. Some researchers have obtained better growth of dermatophyte species at 28°C. [10]

In vitro susceptibility showed that griseofulvin had the lowest MIC followed by itraconazole and terbinafine. All the isolates showed very high MIC against fluconazole. A study conducted in Brazil showed the similar findings, itraconazole and terbinafine with low MIC and fluconazole with high MIC. [10] Highest MIC for itraconazole against the dermatophytes was 8µg/ml (20%), whereas other studies reported it to be 1µg/ml [6] and 4µg/ml. [8,10] Similarly, highest MIC for fluconazole was 64µg/ml (33.3%); terbinafine was 16µg/ml (3.3%) and griseofulvin was 4µg/ml (13.3%). Other studies reported highest MIC for fluconazole to be 32 µg/ml [10] and 64 µg/ml; [8] terbinafine to be 1µg/ml [10] and 4µg/ml; [6] griseofulvin to be 8µg/ml [10] and 16µg/ml.[8]

T. mentagrophyte isolates were mostly susceptible to griseofulvin (MIC_{50} - 0.25µg/ml & MIC_{90} - 4µg/ml) and terbinafine (MIC_{50} - 0.25µg/ml & MIC_{90} - 4µg/ml). Contrast to our finding, Bhatia et al. reported itraconazole as the most effective drug (MIC_{50} - 0.1252µg/ml) and terbinafine as the least effective drug (MIC_{50} - 0.52µg/ml) for *T. mentagrophyte*.[6] For *T. tonsurans* the most effective drug was griseofulvin (MIC_{50} - 1µg/ml & MIC_{90} - 2µg/ml); fluconazole, itraconazole and terbinafine were not equally effective (fluconazole: MIC_{50} - 32µg/ml & MIC_{90} - 64µg/ml, itraconazole: MIC_{50} - 2µg/ml & MIC_{90} - 8µg/ml, and terbinafine: MIC_{50} - 1µg/ml & MIC_{90} - 8µg/ml). A study from Nigeria reported *T. tonsurans* with low MIC_{50} value (terbinafine 0.07 µg/ml, itraconazole 0.01µg/ml and fluconazole 1µg/ml and griseofulvin 2µg/ml). Further, the same study reported similar low MIC_{50} value of antifungal drugs against other dermatophytes.[8] *T. schoenleinii* has responded mostly to itraconazole followed by griseofulvin (MIC_{50} – 0.5µg/ml & MIC_{90} - 2µg/ml and MIC_{50} – 0.5µg/ml & MIC_{90} - 4µg/ml respectively); it showed high MIC to fluconazole followed by terbinafine (MIC_{50} – 16µg/ml & MIC_{90} – 64µg/ml and MIC_{50} – 4µg/ml & MIC_{90} – 8µg/ml respectively). *T. rubrum* was highly responding to griseofulvin and itraconazole (MIC_{50} – 0.03125µg/ml & MIC_{90} – 0.0625µg/ml and MIC_{50} – 0.0625µg/ml & MIC_{90} – 0.125µg/ml respectively). *T. rubrum* too was found to be highly non responsive to fluconazole (MIC_{50} – 16µg/ml & MIC_{90} – 64µg/ml). *T. rubrum* showed MIC_{50} for terbinafine (1 µg/ml), which was found to be similar to a study conducted by Bhatia et al. (0.5µg/ml).[6] Study done by us suggests that fluconazole should be completely stopped and griseofulvin should be re-introduced for the treatment of dermatophytosis in this region. *T. tonsurans* should not be treated with itraconazole and terbinafine, whereas, *T. mentagrophyte* and *T. schoenleinii* can be treated with the combination of itraconazole and terbinafine.

Dermatophytes are showing increasing non responsiveness to the most commonly used antifungal agents i.e. itraconazole and terbinafine. The MIC_{50} and MIC_{90} observed in the present study based on standard protocol M38-A2 of CLSI 2008 might serve as reference for further studies covering large number of isolates from different geographic regions. Also, such studies might reflect on the acquisition of drug resistance among isolates of dermatophyte species based on MIC values.

The present study highlights the drug sensitivity pattern of dermatophytes in this part of the country. Dermatophytosis is caused by multiple species, which cannot be pin point on the clinical ground and different species show different drug susceptibility pattern, which thereby suggest multidrug therapy could be a better option for the management of a case. Other measures that can be taken care for the reduction of antifungal resistance are to avoid misuse, over use, under dose, irregularities during the course of treatment.

CONFLICT OF INTEREST: There is no conflict of interest between any of the authors.

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