Dermatophytic infection and *in vitro* activities of antifungal drugs against dermatophytes in rural India

Dharmendra Prasad Singh,[1] Ankita Sharma,[1] Rajesh Kumar Verma,[1] Sweta S. Kumar,[2] Satender Saraswat[1]

[1]Department of Microbiology, and [2]Department of Dermatology, Uttar Pradesh University of Medical Sciences (UPUMS), Saifai, Etawah, Uttar Pradesh, India

Summary

Background. Increase in resistance to conventional antifungals renders the need for antifungal sensitivity testing in dermatophytes. The present study aimed at determining the prevalence of dermatophytic infections and their susceptibility pattern in a rural healthcare facility.

Methods. Patients with suspected dermatophytosis attending the dermatology outpatient department were enrolled in the study. Specimen collection for mycological examinations was done. *In-vitro* antifungal sensitivity testing was performed as per the Clinical and Laboratory Standard Institute M38-A2 (2008) standards with broth microdilution method.

Results. Onychomycosis was the commonest (41.9%) presentation. Dermatophytic prevalence based on culture was 110 (70.9%). The commonest species was *Trichophyton rubrum* (36.8%). Terbinafine was found to be the most effective drug, followed by ketoconazole and itraconazole.

Conclusions. Antifungal sensitivity in dermatophytic infections should be made a routine in tertiary healthcare facilities as we are already witnessing the rampage of emerging fungal infection– Mucormycosis, in the nation and worldwide.

Correspondence: Satender Saraswat, Department of Microbiology, Uttar Pradesh University of Medical Sciences (UPUMS), Saifai, Etawah–206130, Uttar Pradesh, India.
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Key words: Dermatophytes; itraconazole; ketoconazole; minimum inhibitory concentration; terbinafine.

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Introduction

Disease caused by fungi in warm-blooded animals is known as mycoses. Dermatophytosis is the most common superficial fungal infection caused by dermatophytes, which invades the keratin of skin, hair, and nails for nutrition (4). Twenty to twenty-five percent of the world's population is suffering from superficial mycoses (13). It is also known as ringworm which is caused by *Trichophyton, Epidermophyton,* and *Microsporum*. Skin, hair, and nails are mainly infected by *Trichophyton* species, *Microsporum* species mainly infects skin and hair and not nails and *Epidermophyton* species infects skin and nails (4).

This infection is more prevalent in tropical and subtropical countries, like India, where heat and humidity are high for most of the year. Infection is usually transmitted by direct contacts or through fomites, such as contaminated clothes, hairbrushes, furniture, theatre seats, bed linens, and shower stalls (2). In India, this infection is prevalent in all age groups of both sexes. Dermatophytosis constitutes 16 to 75% of all mycological infections. Predisposing factors include overcrowding, low socioeconomic status, unhygienic living conditions, outdoor work, increased physical activity, and excessive sweating (18).

Diagnosis of the infection is based on both history and clinical examination. Diagnosis is confirmed by potassium hydroxide (KOH) microscopy and culture examination. Clinical diagnosis needs to be supported by laboratory diagnosis (10). Culture examination is very important for the identification of etiological agents. This is important for the choice of treatment for the nail and skin infection, caused by dermatophytic and non-dermatophytic fungi which can be resistant to the usual dosage of therapy (5).

There are different methods for determining antifungal susceptibility, *i.e.*, broth microdilution, agar dilution, E-test, and disc-diffusion methods. MIC is defined as the lowest concentration of an antimicrobial at which it inhibits the visible growth of a microorganism after overnight incubation. MICs are mainly used to observe the resistance pattern and as a tool of research for determining the in-vitro activity of antimicrobials in diagnostic laboratories (1).

MICs for filamentous fungi, including dermatophytes, are determined according to Clinical and Laboratory Standards Institute (CLSI) approved protocol M38-A2 (2008) (15). The determination of resistance patterns of isolated fungal strains helps clinicians in better management of disease and selecting appropriate therapeutic options (15).

For the treatment of the infection, topical and systemic antifungal agents are used. Topical applications are suitable in the eradication of organisms in mild cases, and for severe cases like tinea capitis and tinea unguium, administration of systemic antifungals is needed (19). To increase the cure rate, combined therapy of topical and oral antifungals, along with anti-inflammatory drugs has been used (11).

This study aims to determine the clinical pattern of dermatophytes in patients and the *in-vitro* activity of antifungal agents which are most commonly used to treat dermatophytic infection.

Materials and Methods

Patients with suspected dermatophytosis attending the outpatient department at Uttar Pradesh University of Medical Sciences (UPUMS), Saifai, Etawah, Uttar Pradesh were enrolled in the study from January 2019 to July 2020. Prior consent, followed by detailed history and clinical examination were recorded. This study was approved by the Institutional Ethics Committee with the Ethical Clearance Code - 143/2018.

Superficial fungal infection was suspected in presence of a lesion with a central clear zone, elevated border which was red and had scales, sometimes vesicles, on the border of the lesion. Tinea corporis was suspected when there was a circular plague with a well-defined border, and Tinea cruris was suspected with erythematous plague and pruritus. Tinea pedis (Figure 1) was suspected with macerated areas in the webs, and chronic dry scaly hyperkeratosis on the sole. Tinea manuum was considered when erythema was present with mild scaling on the dorsum, and chronic, dry scaly hyperkeratosis on the palm. Onychomycosis (Figure 2) was suspected when there was distal hyperkeratosis along with chalky dull yellow debris under the nail bed and the nail plate was brittle.

Samples

360 patients were suspected to have a superficial fungal infection. Skin scrapings, nail clippings, and hair roots were taken as specimens. Before taking the samples, the area was cleaned with 70% alcohol to avoid contamination. The skin scrapings were taken from the edges of the lesion with the blunt side of the scalpel. Clippings were taken from infected nails and hair roots were taken by plucking with sterile forceps.

Methodology

KOH microscopy (Figure 3) and culture on Sabouraud's Dextrose Agar (SDA) (Figure 4) were done for all the samples for the presence of fungal elements and fungal growth, respectively. All the KOH-positive and/or culture-positive samples were analysed further.

All the specimens were inoculated on SDA with cycloheximide (0.05g/L) and chloramphenicol (0.005g/L) (HiMedia™ Laboratories Pvt. Ltd., Mumbai, India). Test tubes were incubated at 25-28°C in a Biological Oxygen Demand (BOD) incubator for 4 weeks before labelling it negative. Species identification was done by colony morphology and microscopy on lactophenol-cotton-blue (LPCB) mount (Figure 5). If there was no growth for 4 weeks, it was considered negative. The urease test was used to differentiate between *Trichophyton rubrum* and *Trichophyton mentagrophyte*.

Antifungal agents

Three antifungal agents– itraconazole, ketoconazole and terbinafine, in powdered form were used. Concentration of ketoconazole was 16 µg/mL, while that of itraconazole and terbinafine was 0.5 µg/mL each.

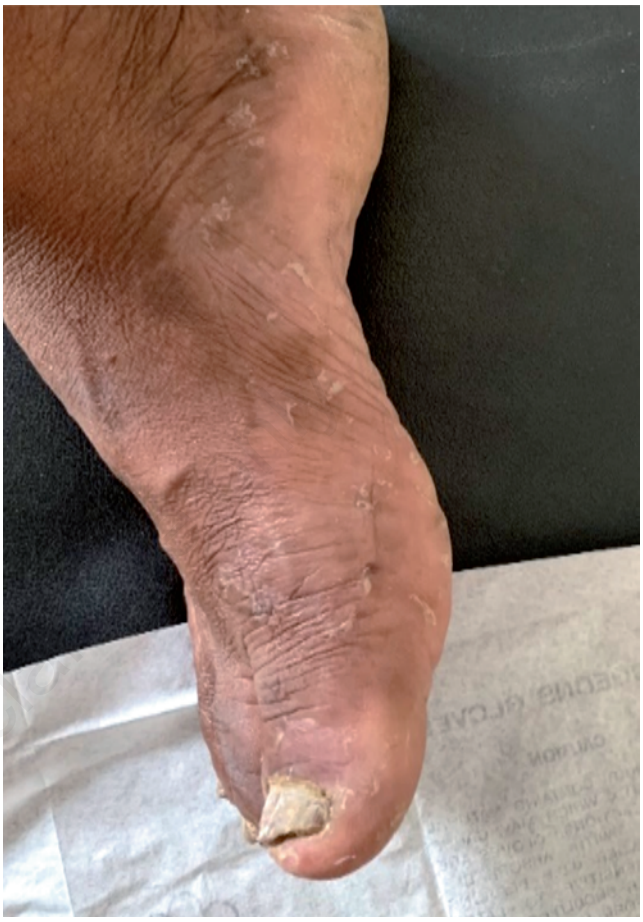

Figure 1. Partially treated case of Tinea pedis.

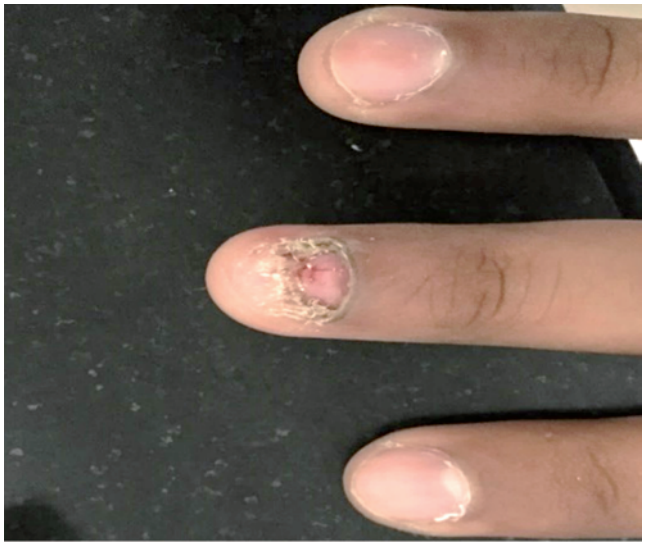

Figure 2. Total dystrophic onychomycosis in a patient.

Antifungal susceptibility testing (broth microdilution method)

It was done according to the M38-A2 approved protocol of CLSI (2008) for filamentous fungi. Firstly, stock solutions of all the drugs, *i.e.*, itraconazole, ketoconazole, and terbinafine, were prepared in dimethyl sulfoxide (HiMedia™ Laboratories Pvt. Ltd., Mumbai, India). Then dilutions were prepared from a stock solution in Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine and without sodium bicarbonate (HiMedia™ Laboratories Pvt. Ltd., Mumbai, India). The pH of the solution was maintained at 7 by adding 1N Sodium hydroxide [HiMedia™ Laboratories Pvt. Ltd., Mumbai, India](Figure 6).

Inoculum preparation

The known species of dermatophytes (7–8 days old) grown on Potato Dextrose Agar (PDA) slants at 30°C, were used to prepare inoculums. A loopful of growth was taken in screw-capped test tubes containing 5 mL of 0.85% normal saline, which were then vortexed to suspend the spores. Then, these tubes were allowed to stand for 10-15min for the heavier particles to sediment. The upper clear suspension thus obtained, was transferred to another test tube and the optical density was adjusted at 0.5 McFarland standards. Final cell density was set between $2x10^3$ to $6x10^3$ colony forming units (CFU) per mL.

MIC testing

96-well U-shaped microtiter sterile plate was taken and marked T1 to T12 horizontally. 100µL of each prepared drug dilution was added from T1 to T10. Then 100µL of prepared inoculums were added from T1 to T10 in all the wells, accordingly. The inoculum was added to T11 without any drug to act as growth control, while in T12 only RPMI medium was added without drug to act as a medium control. The trays were rocked thoroughly to get an even suspension of the inocula. The Microtiter plate was covered with the lid and kept at 35°C for 4-5 days. The reading was taken every 24 hrs with the help of a reading mirror. The MIC value was considered at the point at which no growth was detected in the wells visually (80-100% inhibition).

Data analysis

Data was analysed and evaluated statistically using IBM SPSS STATISTICS version 20.

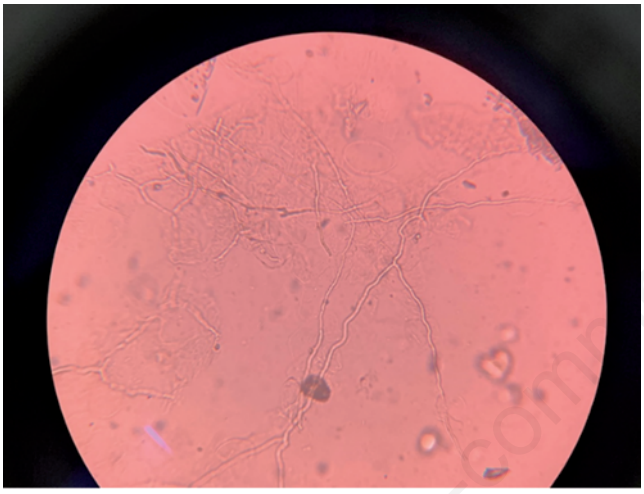

Figure 3. Potassium hydroxide (KOH) mount from the skin of a case of Tinea pedis.

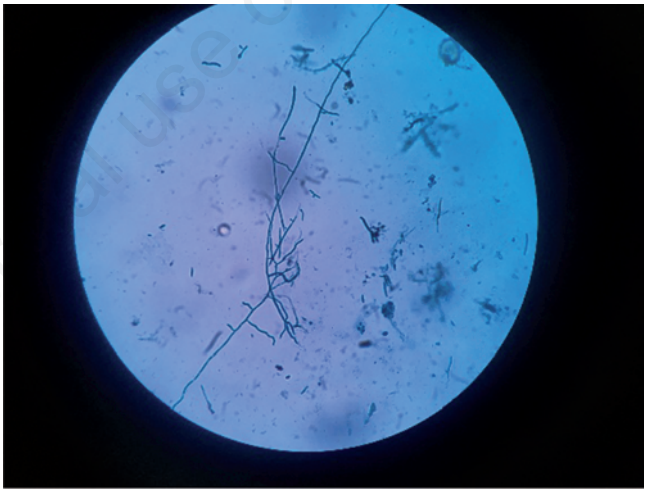

Figure 5. Trichophyton rubrum on lactophenol-cotton-blue (LPCB) mount.

Figure 4. Growth of Trichophyton rubrum – obverse and reverse, on plain Sabouraud's Dextrose Agar (SDA).

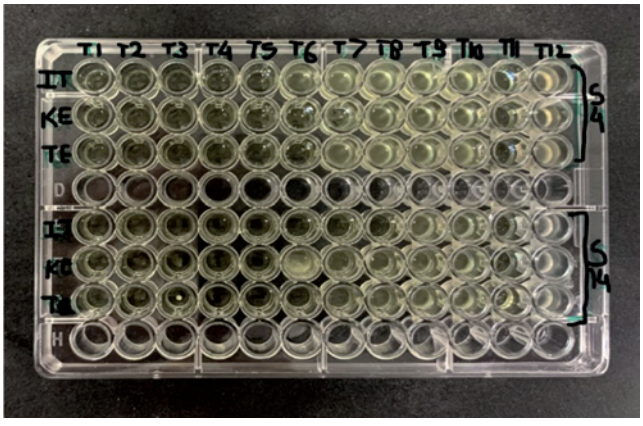

Figure 6. In-process labelled broth microdilution microtiter plate.

Results

Out of 360 clinically diagnosed patients of superficial fungal infection, 223(61.9%) were males and 137 (38.1%) were females with a male-female ratio of 1.62:1. The age of patients ranged from 2 to 70 years with a mean of 29.35±15.14yrs. The commonest age group involved was 21-30yrs with 67 (30.0%) males and 39 (28.5%) females (Table 1). Specimens from different sites were collected, *i.e.*, skin, nails, and hair. Overall, the majority of specimens were of skin 175 (48.6%), followed by nails 151 (41.9%) and hair 34 (9.4%).

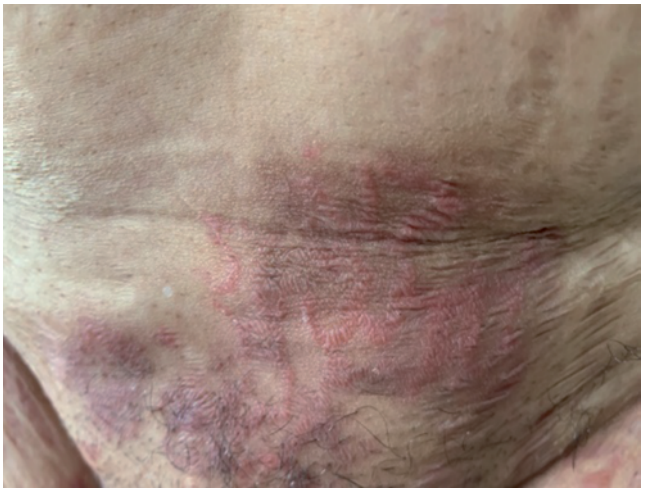

Figure 7. Erythematous lesions in a case of Tinea corporis.

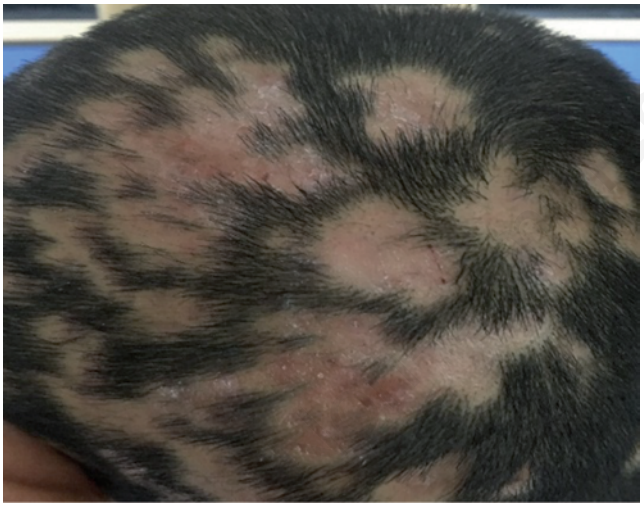

Figure 8. Case of Tinea capitis with patchy lesions of alopecia.

Onychomycosis was seen in 151 patients (41.9%), followed by tinea corporis 115 (31.9%) and tinea capitis 34 (9.4%) as depicted in Table 2. Most of the patients (185, 51.4%) presented with a clinical history of less than 3 months, followed by 4-6 months (25.6%), and 10-12 months 12.2%. Those with a history of more than 12 months were 6.9% whereas the least number of patients aged 7-9 months (3.8%).

Only 50 (13.9%) patients had a history of contact. One hundred and forty-three (39.7%) patients had a history of treatment, among which, 41 (28.7%) were on regular treatment and 102 (71.3%) skipped treatment often. Eighty-one (56.6%) of the patients on treatment were on systemic treatment while 57 (39.8%) were on topical treatment.

KOH examination was positive for fungal elements in 187 (51.9%) patients; culture results showed fungal growth in 155 (43.0%) inoculated samples, and 95 (26.4%) samples were positive for both KOH and culture. Twenty-nine (8.1%) of the inoculated samples were contaminated (Table 3). Out of 155 culture-positive cases, dermatophytes were 110 (70.9%) and non-dermatophytes were 45 (29.1%). Among dermatophytes, the most common species was *Trichophyton rubrum* (57; 36.8%), followed by *Trichophyton mentagrophytes* (36; 23.2%), and *Trichophyton violaceum* (7; 4.5%) as seen in Table 4. *Candida* spp. 22 (14.1%), followed by *Fusarium* spp. and *Aspergillus flavus* (8; 7.01% each), *Geotrichium candidum* (3; 1.9%) were commonest among the non-dermatophytes (seen in most cases of Onychomycosis).

Antifungal sensitivity was done on all the isolated dermatophytes using three antifungals *i.e.*, itraconazole, terbinafine, and ketoconazole, by determining their MIC_{50} (minimum concentration that inhibits 50% of isolates), MIC_{90} (inhibits 90% of isolates), geometric mean (GM) and MIC range as illustrated in Table 5.

For *T.mentagrophytes*, MIC_{50}, MIC_{90}, GM, and MIC range for itraconazole was 0.25µg/mL, 0.50µg/mL, 0.245µg/mL and 0.062-0.5µg/mL, respectively. For terbinafine, it was 0.125µg/mL, 0.475µg/mL, 0.167µg/mL and 0.031-1.0µg/mL, respectively. For ketoconazole, it was 0.125µg/mL, 1.3µg/mL, 0.328µg/mL and 0.031-2.0µg/mL, respectively.

For *T.rubrum*, MIC_{50}, MIC_{90}, GM, and MIC range for itraconazole was 0.25µg/mL, 1.0µg/ mL, 0.374µg/mL and 0.062-1.0 µg/mL, respectively. For terbinafine, 0.125µg/mL, 1.0µg/mL, 0.311µg/mL and 0.031-2.0µg/mL. For ketoconazole, 0.125µg/mL, 1.0µg/mL, 0.362µg/mL and 0.031-1.0 µg/mL, respectively.

For *T.violaceum*, MIC_{50}, MIC_{90}, GM, and MIC range for itraconazole was 0.25µg/mL, 0.25µg/mL, 0.25µg/mL and 0.062-1.0

Table 1. Age and gender-wise distribution of study participants.

Age group	Male (n=223)		Female (n=137)		Total (n=360)	
	Number	Percentage	Number	Percentage	Number	Percentage
Up to 10 years	16	7.2	15	10.9	31	8.6
11-20 years	59	26.5	35	25.5	94	26.1
21-30 years	67	30.0	39	28.5	106	29.4
31-40 years	28	12.6	17	12.4	45	12.5
41-50 years	27	12.1	18	13.1	45	12.5
51-60 years	16	7.2	11	8.0	27	7.7
>60 years	10	4.5	2	1.5	12	3.3

µg/mL, respectively. For terbinafine, 0.125µg/mL, 1.0µg/mL, 0.311µgmL and 0.031-2.0µg/mL. For ketoconazole, 0.125µg/mL, 1.0µg/mL, 0.362µg/mL and 0.031-1.0 µg/mL, respectively.

For *T.verrucosum*, MIC_{50}, GM and MIC range for itraconazole was 0.5 µg/mL, 0.417 µg/mL, and 0.25-0.5 µg/mL. For terbinafine, MIC_{50}, MIC_{90}, GM and MIC range were 0.125 µg/mL, 0.125 µg/mL, 0.125 µgmL and 0.125 µg/mL. For ketoconazole, MIC_{50}, GM, MIC range were 0.031, 0.041 and 0.031- 0.062 µg/mL.

For *T.tonsurans*, MIC_{50}, MIC_{90}, GM and MIC range for itraconazole were 0.25 µg/mL, 0.25 µg/mL, 0.25 µg/mL and 0.25 µg/mL. For terbinafine, MIC_{50}, GM and MIC range were 0.1875 µg/mL, 0.187 µg/mL and 0.125-0.25 µg/mL. For ketoconazole, MIC_{50}, MIC_{90}, GM and MIC range were 0.031 µg/mL, 0.031 µg/mL, 0.031 µg/mL and 0.031 µg/mL.

For *E.floccosum*, MIC_{50}, GM and MIC range for itraconazole were 0.375 µg/mL, 0.375 µg/mL, 0.25-0.25 µg/mL. For terbinafine, MIC_{50}, MIC_{90}, GM and MIC range were 0.5 µg/mL, 0.5 µg/mL, 0.5 µg/mL and 0.5 µg/mL. For ketoconazole, MIC_{50}, MIC_{90}, GM and MIC range were 0.125 µg/mL, 0.125 µg/mL,0.125 µg/mL and 0.125 µg/mL.

For *M.gypseum*, MIC_{50}, GM and MIC range for itraconazole were 0.125 µg/mL, 0.167 µg/mL and 0.125-0.25 µg/mL. For terbinafine, it was 1 µg/mL, 1.17 µg/mL and 0.5-2.0 µg/mL, respectively. For ketoconazole, 0.125 µg/mL, 0.104 µg/mL, and 0.062-0.125 µg/mL, respectively.

Discussion

A total of 360 cases were diagnosed to have superficial mycoses, commonly affecting the age group of 21-30 yrs. The study shows that skin was the commonest site of infection, followed by nails and hair. Studies by Mishra *et al*. (17) and Goldstein *et al*.(8) revealed that the commonest presentation was that of Tinea corporis (Figure 7) followed by Tinea cruris and Onychomycosis. However, in our study, onychomycosis was the commonest clinical presentation, followed by tinea corporis, tinea capitis (Figure 8), and tinea cruris.

In the study by Suman *et al*.(29) males were affected more than females. This result concords with our study. This could be due to their involvement in outdoor activities including farming and labour work. This could also be attributed to the rural locality of our healthcare facility, providing higher exposure to the male population.

A study conducted by Kumar *et al*. (12) found the duration of symptoms to be greater than 3 months in 53.3%, 1-3 months in 33.7% cases, and less than 1 month in 13% of cases. While in our study, chronic infection of more than 6 months was found in 48.6% of patients due to irregular treatment, application of topical steroids (which only reduces inflammation and pruritus), and inadequate doses of anti-fungal medication, when evaluated.

In the studies conducted by Manjunath *et al*. (16) and Lavanya *et al*.(14) *T.rubrum* was the commonest isolated dermatophyte followed by *T.mentagrophytes*. Similar results were found in our study. Grover *et al*. (9) found that the prevalence of non-dermatophytic infections was 34%, which is similar to our result of 29.1%.

The distribution pattern of dermatomycoses and their causative agents varies with geographical area, the community where the person is living, socioeconomic status, and hygiene habits as people who live in overcrowded areas and have low socioeconomic status are more susceptible to the infection.

Dermatophytosis is the widespread superficial fungal infection in humans and domestic animals, many new antifungal agents are being introduced to treat this condition. Due to the introduction of a wide range of new antifungals, there is the emergence of more resistant organisms to antifungal agents like amphotericin B, azole groups, etc., due to which antifungal susceptibility testing becomes important for the identification of resistant strains and also helps in better management of diseases caused by them.

There are many techniques used to determine antifungal susceptibility, *e.g*., disk diffusion, broth macro and microdilution techniques, colorimetric microdilution methods, and E-test. The M38-A2 protocol by CLSI (2008) is used to determine antifungal susceptibility for filamentous fungi. In our study, terbinafine and ketoconazole have lower mean MIC values as compared to itraconazole. This suggests that terbinafine and ketoconazole are more effective as compared to itraconazole. Many other studies reported low MIC values of terbinafine and ketoconazole.

In our study, *T. mentagrophytes* and *T.rubrum* isolates were more susceptible to both terbinafine and ketoconazole with lower MIC_{50} values, *i.e*., 0.125µg/mL whereas this value was recorded as 0.25µg/mL for itraconazole. Both species exhibited similar susceptibility to the drugs. Similarly other species like *T.violaceum, T.verrucosum. T.tonsurans* they were more susceptible to terbinafine and ketoconazole followed by itraconazole.

Whereas *Epidermophyton floccosum* was more susceptible to

Table 2. Site of fungal infection among the study cases.

Clinical presentation	Number	Percentage
Onychomycosis	151	41.9
Tinea corporis	115	31.9
Tinea capitis	34	9.4
Tinea cruris	27	7.5
Tinea faciei	15	4.1
Tinea mannum	10	2.7
Tinea pedis	6	1.6
Tinea ungium	2	0.5

Table 3. Prevalence of dermatophytic infection based on potassium hydroxide (KOH) mount and fungal culture (n=360).

Diagnosis	Number	Percentage
KOH positive	187	51.9
Culture positive	155	43.0
Both KOH and culture positive	95	26.4
Contaminant on culture	29	8.1
Both negative	84	23.3

Table 4. Distribution of isolated dermatophytes among the study cases (n=155).

Dermatophytes	Number	Percentage
Trichophyton mentagrophytes	36	23.2
Trichophyton rubrum	57	36.8
Trichophyton violaceum	7	4.5
Trichophyton verrucosum	3	1.9
Microsporum gypseum	3	1.9
Trichophyton tonsurans	2	1.3
Epidermophyton floccosum	2	1.3

Table 5. Determination of MIC values of antifungal drugs against dermatophyte species by broth microdilution method.

Species	MIC Value	Itraconazole	Terbinafine	Ketoconazole
Trichophyton mentagrophytes	MIC_{50}	0.25	0.125	0.125
	MIC_{90}	0.50	0.475	1.30
	GM	0.245	0.167	0.328
	MIC range	0.062-0.50	0.031-1.0	0.031-2.0
Trichophyton rubrum	MIC_{50}	0.25	0.125	0.125
	MIC_{90}	1.0	1.0	1.0
	GM	0.374	0.311	0.362
	MIC range	0.062-1.0	0.031-2.0	0.031-1.0
Trichophyton violaceum	MIC_{50}	0.25	0.062	0.062
	MIC_{90}	0.25	0.25	0.25
	GM	0.25	0.134	0.129
	MIC range	0.125-0.5	0.031-0.25	0.031-0.25
Trichophyton verrucosum	MIC_{50}	0.5	0.125	0.031
	MIC_{90}	-	0.125	-
	GM	0.417	0.125	0.041
	MIC range	0.25-0.5	0.125	0.031-0.062
Trichophyton tonsurans	MIC_{50}	0.25	0.1875	0.031
	MIC_{90}	0.25	-	0.031
	GM	0.25	0.187	0.031
	MIC range	0.25	0.125-0.25	0.031
Epidermophyton floccosum	MIC_{50}	0.375	0.50	0.125
	MIC_{90}	-	0.50	0.125
	GM	0.375	0.50	0.125
	MIC range	0.25-0.5	0.5	0.125
Microsporum gypseum	MIC_{50}	0.125	1	0.125
	MIC_{90}	-	-	-
	GM	0.167	1.17	0.104
	MIC range	0.125-0.250	0.5-2.0	0.062-0.125
Total	MIC_{50}	0.250	0.125	0.125
	MIC_{90}	0.950	1.0	1.0
	GM	0.317	0.272	0.258
	MIC range	0.062-1.0	0.031-2.0	0.031-2.0

ketoconazole having a lower MIC_{50} value, *i.e.*, 0.125µg/mL, followed by itraconazole with MIC_{50} value, i.e., 0.375 µg/mL then terbinafine with MIC_{50} value, *i.e.*, 0.50 µg/mL. For *Microsporum gypseum*, they were equally susceptible for both ketoconazole and itraconazole followed by terbinafine.

On comparing MICs of the three antifungals based on the t-test, p-value between 'itraconazole and terbinafine and 'itraconazole and ketoconazole' is 0.005 so statistically, there is a significant difference among their sensitivity. Whereas between 'terbinafine and ketoconazole' P-value is 0.63 which shows that there is no significant difference in their sensitivity.

Bhatia *et al*. (3) stated that itraconazole and ketoconazole had lower mean MIC values as compared to terbinafine. Studies by Fernandez *et al*. (6) and Ghannoum *et al*. (7) also report the lower MIC values of these antifungal agents. While in our study, terbinafine was found to be the most sensitive drug. The second most sensitive drug was ketoconazole, followed by itraconazole. Thus, our results are in contrast to these studies.

Conclusions

Trichophyton rubrum was the commonest species among the dermatophytic infection in cases of superficial fungal infections with the most affected age group of 21-30 years. Males were more susceptible. Among the tested antifungals, terbinafine was the most sensitive drug. Antifungal susceptibility testing should be employed as a routine investigation in healthcare facilities before the prescription of antifungal agents, to avoid the development of resistance in the dermatophytes causing superficial infections. The recurrent dermatophytosis is quite frequently seen these days but cannot be explained alone by the high MIC of antifungal drugs, and further research and study into the topic will be of paramount importance. Dermatophytes are emerging fungi with an increase in the MIC of the most commonly used antifungal agents. The possible reasons could be overt use and abuse of antifungal drugs, favourable environmental conditions for the causative agent, and host factors like poor hygiene, leading to emerging health hazards to the community. This becomes important as we are already viewing the impact and severity of the post-COVID (Coronavirus disease) invasive fungal infections emerging in the nation and worldwide.

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