

Original Article

Resistance & virulence traits in dermatophytes isolated from Mangaluru, India

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Background & objectives: Dermatophytes are accountable for the majority of fungal skin infections globally, affecting 20-25 per cent of the world population. Though not fatal, these infections have significant psychosocial impacts and reduce the quality of life. Prevalence of the infection varies geographically, influenced by factors like social practices, migration and climate. Understanding the pathogenicity of dermatophytosis including virulence factors and drug resistance, is necessary to identify factors that predispose recalcitrance.

Methods: A prospective hospital-based study was carried out in the dermatology departments of two tertiary care hospitals in Mangaluru, India from November 2018 to March 2021. We included 93 individuals of recalcitrant tinea infections, and excluded those with diabetes or those under immunosuppressive therapy. Skin scrapings from lesions were cultured, and DNA extracted for ITS sequencing. All samples were processed for antifungal susceptibility testing, and mutation analysis in squalene epoxidase gene for representative isolates and virulence factor assays.

Results: Of 93 clinically diagnosed individuals with dermatophytosis, dermatophytes were recovered in 70.96 per cent samples, with *Trichophyton mentagrophytes* complex being the most common agent. Antifungal susceptibility testing showed high MICs for fluconazole, terbinafine and itraconazole in several isolates, indicating *in-vitro* resistance. Mutation analysis for six isolates revealed missense mutations in the squalene epoxidase gene. Virulence activity analysis showed high enzyme production levels among isolates, contributing to their pathogenicity.

Interpretation & conclusions: These findings underscore the complexity of dermatophytosis and emphasize the need for persistent tracking of antifungal resistance patterns and virulence factors. Such insights are vital for developing effective treatment strategies and improving patient outcomes due to rising antifungal resistance.

Key words Antifungal resistance - dermatophytosis - recalcitrant - tinea - trichophyton - virulence

Dermatophytes cause the most prevalent fungal infections and affect up to 25 per cent of the world's ailments globally resulting in superficial fungal skin population. Although not linked to catastrophic results,

it is recognized to have a significant psychosocial impact and decline in quality of life in affected persons. Yet, they seem to be consistently overlooked¹. According to the geographical area, the prevalence of dermatophyte infection varies. The divergence in distribution pattern is linked to military movements, social practices, extensive global travel, immigration, and labour migration². Traditionally, dermatophytosis respond well to a diverse array of topical and oral antifungal medications with quick cure on treatment with short courses of conventional antifungal medications. However, in the last few years, several portions of India have seen a shifting situation of extensive chronic, recurring, and resistant dermatophytosis^{3,4}. Dermatophytosis is widespread in India, primarily due to the favourable climatic conditions characterized by elevated temperatures and humidity levels. Being a tropical nation in a developmental phase, India faces challenges associated with dermatophytosis, exacerbated by economic factors such as poverty, substandard hygiene practices and social issues like overcrowding⁵. Several studies and guidelines have all been conducted in response to this frightening recalcitrant dermatophytosis epidemic to help practicing clinicians combat it more successfully^{6,7}.

The first stage of dermatophytosis involves contact with infected animals and humans or indirectly through contamination, followed by adherence to keratinized tissue reaching the epidermis and penetrating the stratum corneum. Dermatophytes penetrate the skin and produce various virulence factors to promote infection, including enzymatic and non-enzymatic elements. These fungi are often cleared from the skin by a cell-mediated immune response in generally immunocompetent individuals. In contrast, the skin infected by the dermatophyte can be extensively damaged by the invasive nature of the disease, which is associated with considerable local damage of deep tissues and leads to systemic disease in vulnerable patients⁸. The increase in chronic, recurrent and re-infection in sensitive populations is a growing concern with the contributing factors including poor compliance with medication regimen, steroid use, over-the-counter medication use, treatment failure and potential antifungal resistance⁹. Among the associated mechanisms and factors responsible for the pathogenicity of dermatophytosis are the host's immune response, enzymatic and non-enzymatic virulence, drug resistance, and other environmental factors. Pathogenicity, however, has been viewed as multifactorial and independent, making an

understanding of the dynamic processes essential for the effective prevention, diagnosis, and treatment of dermatophytosis. This study aims to determine the prevalent etiological agent and associated resistant determinants in recalcitrant dermatophytosis.

Materials & Methods

This was a hospital-based prospective study conducted in the Dermatology departments, at two tertiary care hospitals in Mangaluru, India (KS Hegde Medical Academy and Father Muller Medical College hospitals), from February 2019 to March 2021. Ethical approval was obtained from the Institutional Ethics Committee of Nitte Central Ethics Committee, KS Hegde Medical Academy and Father Muller Medical College Hospital. Clinical samples of skin scrapings were obtained after informed patient consent. Patients of age >5 yr and all genders visiting the Dermatology Outpatient Department (OPD) during the mentioned study period with recalcitrant tinea infections were included, excluding patients with conditions underlying such as diabetes, immunosuppressive therapy, HIV positive and patients who refused to give consent.

Sample size estimation: The required sample size was calculated according to following assumption: prevalence of the infection at 40 per cent, precision in recovery, prevalence error of five per cent, and confidence level interval of (CI) 95 per cent. The sample size was estimated to be 93 to obtain at least 50 isolates.

Sample collection and processing: The isolates were obtained from individuals diagnosed with tinea cruris, tinea corporis, tinea unguium or tinea faciei. Skin scraping samples were collected from the edges of the lesions in a sterile container. The patient's name, identification number, age, sex, and sample collection date were recorded and were transported immediately to the laboratory. All the specimens were subjected to treatment with 20 per cent potassium hydroxide direct mount examination and cultured on Sabouraud dextrose agar (SDA) and broth (SDB) with cycloheximide (300 mg/L) and chloramphenicol (50 mg/L) at 28°C for a maximum of four wk with routine examination for dermatophytic growth.

Culture identification: Identification was performed based on their growth characteristics^{10,11}. Extraction of genomic DNA was performed according to lyticase buffer-based extraction¹². This extracted DNA was

used as a polymerase chain reaction (PCR) template using primers targeting ITS region⁹. Products of PCR amplification were purified and sequenced by the Sanger sequencing method (Eurofins Genomics Pvt Ltd.). The nucleic acid sequence obtained from forward and reverse primer were trimmed and assessed using Chromas software and then aligned using the Multalin tool and Clustal-Omega. Sequence similarity of >95 per cent and >98 per cent coverage was determined from the best-scoring reference sequence considered by NCBI nucleotide. All the consensus sequences were submitted to NCBI Genbank. MEGA X (version 10.0) software was used to construct phylogenetic trees by Neighbour Joining method with bootstrap tested for 100 replicates for all the obtained sequences and to expand the spectrum, sequences of standard strains from NCBI Nucleotide included.

Antifungal susceptibility testing: Antifungal susceptibility testing was performed using 96-well microtiter plates in RPMI 1640 medium supplemented with two per cent glucose and 0.165 mol/L 3-N-morpholine propane sulfonic acid (MOPS) at pH 7.0 according to EUCAST guidelines relevant during the study period¹³. Cultures were grown on SDA with cycloheximide (300 mg/L) and chloramphenicol (50 mg/L) for 14 days at 28°C and conidial suspension prepared in 5 mL of 0.85 per cent saline to produce a final spore density of $\sim 3 \times 10^5$ CFU/mL, as determined using a haemocytometer. Antifungal agents itraconazole, fluconazole and terbinafine (Sigma-Aldrich, India) were dissolved in respective solvents according to the manufacturer's guidelines and prepared as stock solutions at 6,400 mg/L for all drugs. The exerted concentration ranges for the drugs were 0.0625 to 32 mg/L. Control strains *Trichophyton rubrum* ATCC 28188, *Trichophyton tonsurans* ATCC 28942, *Trichophyton interdigitale* ATCC 9533 and *Aspergillus fumigatus* ATCC 204305 were used as references and read after 96 h of incubation at 28°C. All the experiments were conducted in triplicates. Culture control, antifungal control and media controls were included. MIC endpoints were defined as the lowest antifungal concentration that resulted in 50 per cent inhibition using a spectrophotometer at 600 nm after four days for all drugs according to EUCAST guidelines.

Mutation analysis in squalene epoxidase gene: Mutations in the squalene epoxidase gene sequences were analysed as per Rudramurthy *et al*⁹, 2018.

PCR reactions were conducted with an annealing temperature of 54°C for forward primer- SEF: CCATGTTGTCCTGGGTG and reverse primer- SER: GGGGAGGAGGTAGATGGGTT. Products of PCR amplification were purified and sequenced after quality checks at the service provider (Sanger sequencing, Eurofins Genomics Pvt Ltd.). The nucleic acid sequences derived from the forward and reverse primer cycle sequences were processed using Chromas software (trimming and quality check) and only reads that resulted in a consensus (>99% identity between forward and reverse reads) obtained in Multalin tool were considered for further analysis. Clustal-Omega software (alignment), NCBI BLAST (Similarity identity) and ExPasy translate tools were used to generate amino acid sequences of the squalene epoxidase gene and identify the mutations in the obtained sequence.

Virulence activity: The activity of virulence enzymes such as phospholipase using egg yolk, lipase using tween 80 agar, gelatinase using gelatine agar, protease using casein agar, keratinase using hair perforation assay¹⁴ and non-enzymatic virulence activity of melanin production by quantification¹⁵ were analysed for all the isolates.

Production of phospholipase: Phospholipases help maintain cell membrane functions and act as virulence factors by invading host cells through the hydrolysis of phospholipids into fatty acids and lipophilic substances. Fungal spores were inoculated in a medium containing one per cent peptone, two per cent dextrose, five per cent sodium chloride, 0.05 per cent calcium chloride, two per cent agar and five per cent egg yolk and incubated for one wk. A halo zone of clearance around the colony indicated phospholipase production.

Production of lipase: Lipases are enzymes that hydrolyse carboxyl ester bonds present in triacylglycerols, disrupting skin tissue and aiding pathogen spread. Medium consisting of one per cent peptone, five per cent sodium chloride, 0.05 per cent calcium chloride, two per cent agar and one per cent Tween 80 was inoculated with fungal spores and incubated for one wk. Lipase production was indicated by a zone of precipitation around the colony.

Production of protease: Proteases are degradative enzymes which catalyse the total hydrolysis of proteins. This enzymatic activity not only aids in

establishing infection but also contributes to tissue damage and inflammation, further exacerbating the infection and making it more challenging to treat. The medium containing 0.4 per cent yeast extract, 0.05 per cent potassium dihydrogen phosphate, 0.05 per cent dipotassium phosphate, 0.05 per cent calcium chloride, two per cent agar and eight per cent casein was inoculated with fungal spores and incubated for a week. A halo zone of clearance around the colony indicated protease production.

Production of gelatinase: Gelatinase contributes to virulence through gelatine degradation, derived from collagen present in a broad range of host substrates. The media for screening gelatinase production contained 0.4 per cent yeast extract, two per cent agar and eight per cent gelatine. Following incubation for five days post-inoculation, the plates were flooded with 0.1 per cent Coomassie brilliant blue solution. Clearing around the colony indicated hydrolysis of the gelatine, while the areas of no hydrolysis resulted in a deep blue colour.

Keratinase activity: Dermatophytes produce enzymes like keratinases that allow them to invade the hair and stratum corneum, facilitating the establishment of infection. The hair perforation test was used to screen for keratinase activity. The test was performed by placing fungal spore suspension in 0.85 per cent saline into petri plates, which contained one per cent yeast extract and small pieces of blonde hair strands. After incubation for four wk, they were examined under the microscope at 200x to observe for perforations or erosions on the hair strands.

Biofilm production: The biofilm-producing ability of recalcitrant dermatophytes was determined using 96 well microtiter plates. Using 100 μ l of 10^6 spores/mL suspension in biological triplicates, seeding it onto 96 well-titre plate with 100 μ l of SDB and incubating it at 28°C for four days. After the incubation for 72 h, the titre plates were washed with 0.85 per cent saline three times, followed by treatment with 0.1 per cent crystal violet solution incubated at room temperature for 15 min and washed with 0.85 per cent saline three times. Finally, 95 per cent ethanol was added and incubated for 15 min; the ethanol transferred to a clean 96-well microtitre plate, and OD was measured at 595 nm¹⁶. Isolates were categorized as strong biofilm producers, moderate biofilm producers, weak biofilm producers

and non-biofilm producers using the following formula¹⁶:

- (a) Non-biofilm producers: OD value \leq media control
- (b) Weak biofilm producers: $2 \times$ media control \leq OD value \leq media control
- (c) Moderate biofilm producers: $4 \times$ media control \leq OD value $\leq 2 \times$ media control
- (d) Strong biofilm producers: $4 \times$ media control \leq OD value.

Data availability: The ITS sequences and squalene epoxidase sequences of newly sequenced strains were deposited in GenBank.

Results

Demographic analysis: Ninety-three patients were included in the study as per the sample size estimated. Tinea corporis was the most common presentation in 87.5 per cent (n=81), followed by tinea cruris in 62.5 per cent (n=58), tinea pedis in 16.67 per cent (n=16) and tinea faciei in 8.33 per cent (n=8). The majority of infected sites of patients were the groin in 70.96 per cent (n=66), the abdomen in 37.5 per cent (n=35) and the buttock and leg in 25 per cent (n=23). Multiple site involvement was observed in 83.9 per cent (n=78) of individuals.

Out of 93 clinical skin scraping samples collected from skin samples, the majority, 60.2 per cent (56/93) of samples were from female patients, and 39.8 per cent (37/93) were from male patients, among which 26.9 per cent (26/93) of patients were above 51 yr of age.

On the direct mount of skin scraping using 20 per cent KOH, 76.3 per cent (71/93) of the samples were positive with fungal elements. Dermatophytes were isolated from 70.96 per cent (66/93) of the clinical samples, among which 52.7 per cent (47/93) were recovered from both SDB and SDA, 13.97 per cent (13/93) were isolated from SDB alone, and four per cent (4/93) were isolated from SDA alone.

Identification of dermatophytes: Among the dermatophytes isolated (n=66), 74.2 per cent (n=49) samples showing granular downy surface with yellow-brown reverse pigmentation, hyphae were branched at right angles with spiralling and produced abundant globose to pyriform microconidia were identified as *Trichophyton mentagrophytes* complex. Thirteen

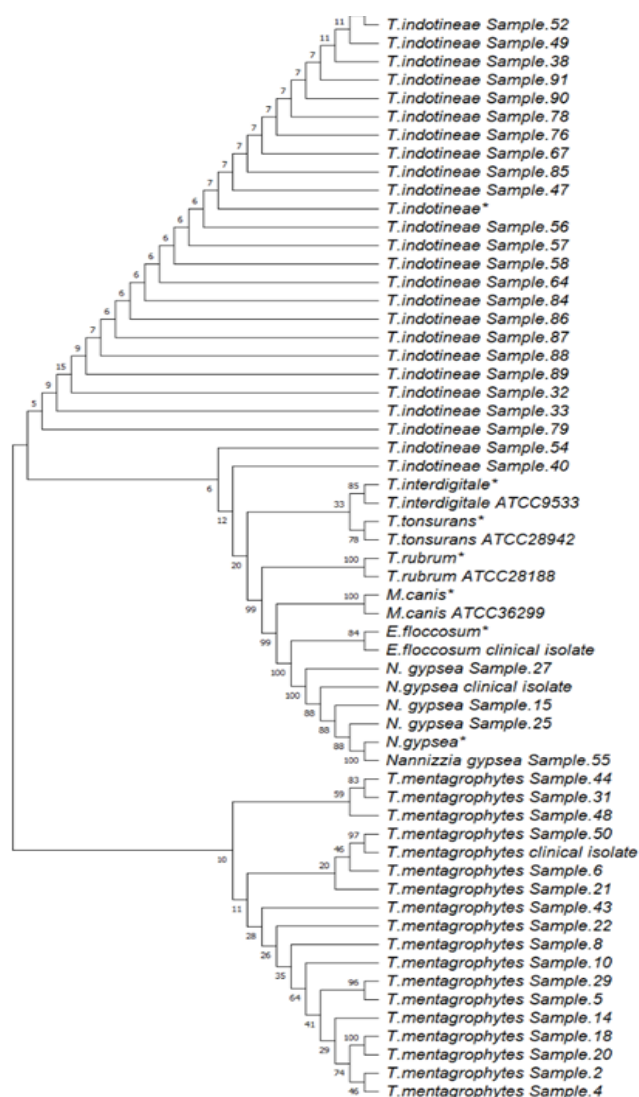


Fig. 1. Phylogenetic tree constructed using the ITS sequence obtained in the study with the reference standard sequences from NCBI.

isolates (19.7%) showing flat-granular surface with brown reverse pigmentation abundant fusiform rough-wall macroconidia divided into 3-6 cells were identified as *Nannizzia gypsea* and four isolates (6.1%) with slightly raised, whitish cream, suede-like to downy, brown to wine-red reverse pigmentation with teardrop shaped abundant microconidia identified as *T. rubrum*.

DNA was extracted using the lysis buffer-based method from standard cultures (n=4), anonymised dermatophyte isolates (n=2) and samples grown in SD media (n=66). DNA of concentrations ranging 200-500 mg/L were obtained, which were then diluted in nuclease free water to obtain working concentrations of 100 mg/L to be used as templates for PCR. All

dermatophytes isolate showed amplification at ~ 600 to 800 base pairs for the ITS region (Supplementary Figure).

Among isolated clinical dermatophytes, sequencing was performed for 42 isolates identified as *T. mentagrophytes* complex, out of which 25 were genotyped as *T. indotineae* (>98% identity), 17 were identified as *T. mentagrophytes*. All the reads were deposited to GenBank, and accession numbers were assigned (Supplementary Table). Phylogenetic trees were constructed using the Neighbour Joining method in MEGA X (version 10.0) with bootstrap tested for 100 replicates (Fig. 1). *T. mentagrophytes* complex clustered together, forming different branches for *T. mentagrophytes*, *T. indotineae* and *T. interdigitale*. However, non-*Trichophyton* species clustered separately.

Antifungal susceptibility testing: Antifungal susceptibility testing was performed for all 66 isolates with itraconazole, fluconazole and terbinafine. All the isolates were categorised into five groups: (i) phenotypically identified as *N. gypsea* (n=13), (ii) phenotypically identified as *T. rubrum* (n=4), (iii) phenotypically identified and genotypically similar to *T. indotineae* (n=25), (iv) phenotypically identified and genotypically confirmed as *T. mentagrophytes* (n=17) and (v) seven isolates phenotypically identified as belonging to *T. mentagrophytes* complex. Terbinafine, an allylamine, exhibited highly potent activity against 25 isolates (38%) at 0.0625 mg/L concentration of the drug and among azoles, itraconazole showed better activity against 23 isolates (35%) at 0.0625 mg/L concentration of the drug. Total of seventeen isolates showed high MIC for itraconazole at 32 mg/L (n=5), 4 mg/L (n=3), 2 mg/L (n=4) and 1 mg/L (n=5), whereas for terbinafine, 32 isolates exhibited highest MIC at 32 mg/L (n=16), 8 mg/L (n=4), 4 mg/L (n=3), 2 mg/L (n=7) and 1 mg/L (n=2), respectively (Fig. 2). However, fluconazole showed poor antifungal activity against the clinical isolates, with 63 isolates showing MIC with more than 1 mg/L of antifungal agent.

Only six representative isolates with MIC >16 mg/L for terbinafine were subjected for squalene epoxidase mutation analysis by comparing the sequences of the squalene epoxidase genes with those of sensitive isolates available on NCBI.

T1189C transition of the open reading frame was noted in four isolates with high terbinafine MICs (>32 mg/L). Additionally, transition of T1243G and T1244C

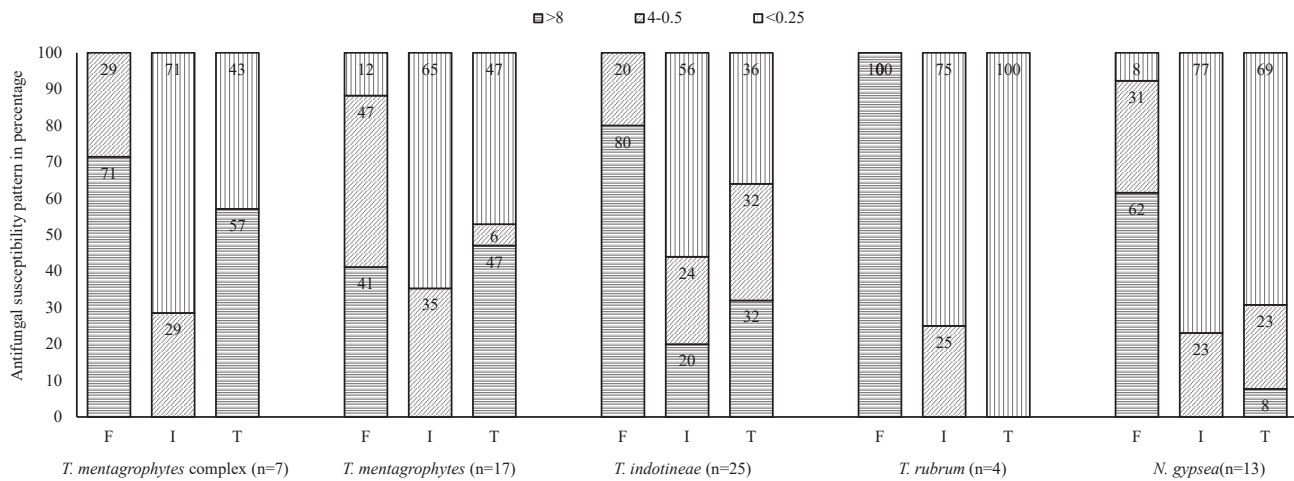


Fig. 2. Antifungal susceptibility of dermatophytes against three antifungal agents. F, fluconazole; I, itraconazole; T, terbinafine.

Table. Salient features of trichophyton isolates used for mutation analysis

| Sample ID | Organism identified by ITS sequencing | ITS accession number | MIC (mg/L) | | | SQ Accession number | Mutation |
|-----------|---------------------------------------|----------------------|------------|------|-----|---------------------|--------------|
| | | | F | I | T | | |
| Sample 2 | <i>Trichophyton mentagrophytes</i> | MN704566 | >32 | 4 | >32 | PP340481 | No |
| Sample 29 | <i>Trichophyton mentagrophytes</i> | MW077535 | 4 | 0.25 | 32 | PP340483 | P397L |
| Sample 31 | <i>Trichophyton indotineae</i> | MT039467 | 4 | 0.5 | 32 | PP340484 | P397L |
| Sample 32 | <i>Trichophyton indotineae</i> | MW494894 | >32 | 32 | 32 | PP340482 | P397L |
| Sample 33 | <i>Trichophyton indotineae</i> | MW077536 | >32 | >32 | >32 | PP340485 | P397L, P415S |
| Sample 38 | <i>Trichophyton indotineae</i> | MT039468 | 8 | 0.25 | 32 | PP340486 | P415I |

ITS, internal transcribed spacer; MIC, minimum inhibitory concentration; F, fluconazole; I, itraconazole; T, terbinafine

was observed in one each isolate. The missense mutation resulted in the change of phenylalanine at the 397th position to leucine (Phe397Leu), at 415th position to serine (Phe415S) or valine (Phe415Val) (Table).

Virulence activity analysis: The virulence activity of lipase, phospholipase, protease and gelatine was determined for all 66 isolates using the plate method by observing the zone of clearance for protease, phospholipase, gelatinase activity and precipitation for lipase activity (Fig. 3). All the isolates were categorised into five groups: (i) phenotypically identified as *N. gypsea* (n=13), (ii) phenotypically identified as *T. rubrum* (n=4), (iii) phenotypically identified and genotypically similar to *T. indotineae* (n=25), (iv) phenotypically identified and genotypically confirmed as *T. mentagrophytes* (n=17) and (v) seven isolates phenotypically identified as belonging to the *T. mentagrophytes* complex. Keratinase activity using hair perforation test was observed in all *T. mentagrophytes* complex, *T. indotineae*, *T. mentagrophytes* and *N. gypsea* isolates. All the isolates (n=7) classified as *T.*

mentagrophytes complex showed lipase, protease, melanin and phospholipase activity. The activity of lipase (n=15), phospholipase (n=13), melanin (n=14) and gelatinase (n=13) were observed to be higher in *T. mentagrophytes*. *T. indotineae* isolates showed activity of phospholipase (n=23), gelatinase (n=22) and lipase (n=21). All *N. gypsea* (n=13) and *T. rubrum* (n=4) isolates showed gelatinase activity. Melanin production was observed in all of *T. rubrum* isolates, a predominant species that produces pigment. Species belonging to *Nannizzia* are also melanin producers with 77 per cent of isolates showing the production in the present study. A higher percentage of *T. mentagrophytes* isolates (82%) produced melanin than *T. indotineae* isolates (68%) (Fig. 4).

Biofilm formation was observed in 59 out of 66 (89%) isolates that were classified as follows: seven isolates were non-biofilm producers (11%), thirty-four isolates were weak producers (52%), twenty-one isolates were moderate producers (31%), and four isolates were strong producers (6%) (Fig. 5). A higher

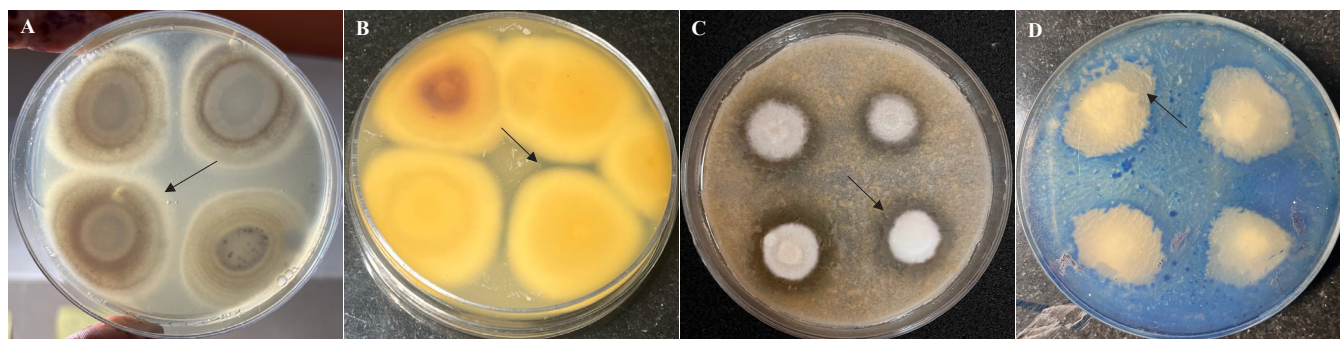


Fig. 3. Representative image for enzymatic activities. (A) Lipase activity observed by precipitation around the colony. (B) Phospholipase activity. (C) Protease activity observed by clear zone around the colony. (D) Gelatinase activity observed by unstained area around the colony.

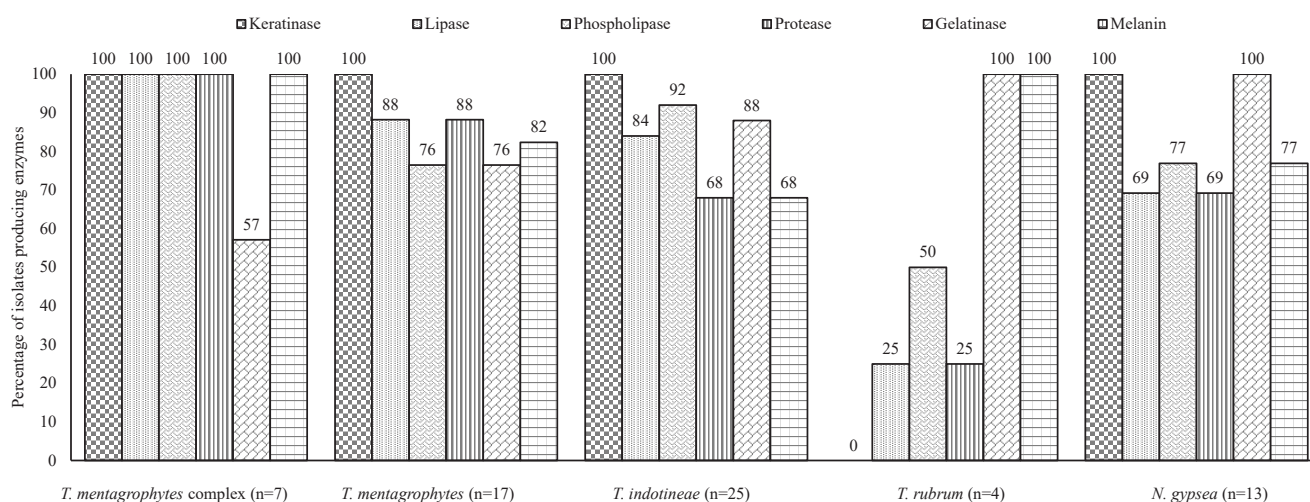


Fig. 4. Graph representing the number of isolates capable of producing virulence enzymes expressed in percentage.

number of *T. indotineae* isolates produced biofilm than the other species.

Discussion

In the present study, 93 samples of clinically diagnosed recalcitrant dermatophytosis involving the skin were evaluated for laboratory diagnosis. Clinical presentations showed that tinea corporis (87.10%) was the most common clinical condition, followed by tinea cruris (70.97%); similar report of the presentation has been reported in other parts of the country¹⁷⁻¹⁹; however, presentation varies depending on the geographical area¹⁸. Direct microscopic KOH mount was positive with the presence of fungal hyphae in 76.3 per cent, and culture was positive in 70.96 per cent of cases. Similar culture recovery was observed by other studies (65-75%)^{20,21}. Dermatophytosis was observed oft-times in females than in males with a female male ratio of 1.5:1. This could be because female patients visited the hospital more frequently than male subjects. Contrary

to our study, other studies from India^{22,23} reported a high occurrence in males. Most of the patients in this study belonged to the age group of more than 50 years. The higher prevalence in this group may be attributed to various factors. Elderly individuals tend to have compromised immune function, experience anatomical and functional changes, and are often exposed to infectious agents, all of which can contribute to their susceptibility to infections²⁴.

Among dermatophytes isolated in the present study, *T. mentagrophytes* complex was the predominant (74.24%) dermatophyte isolated and *T. rubrum* (6.06%) was present in the least number of isolates in concordance with observations from other regions^{21,25}, however in the past decades the *T. rubrum* was the most common causative organism responsible for dermatophytoses^{26,27}. Genotypically 25 isolates identified as *T. mentagrophytes* complex had similarity with *T. indotineae* as per ITS region; however, these isolates hydrolysed urease phenotypically, which

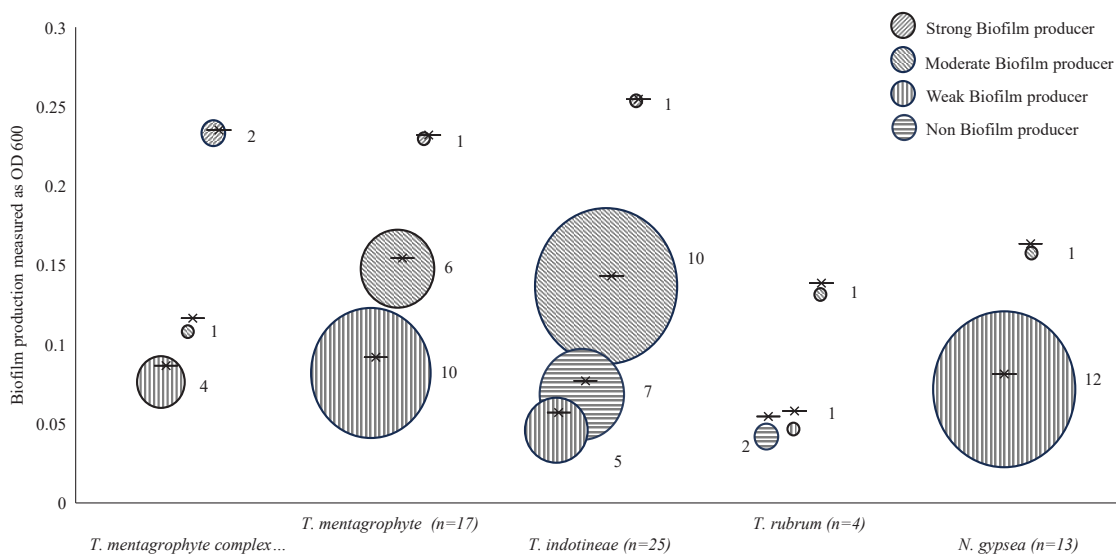


Fig. 5. Ability of the dermatophytes to produce biofilm expressed as optical density measurement. The Y axis co-ordinate represents the average biofilm density, and the size of the bubble is relative to the number of isolates. Graph created in Microsoft Excel 2019.

contradicts the results presented by Kano *et al*²⁸. The controversy regarding the taxonomic labelling of *T. mentagrophytes* type VIII and *T. indotineae* (alternative name to *T. mentagrophytes* type VIII) bears significant complications for mycology, dermatology, and public health. While some argue for the status of *T. indotineae* as a distinct species, powerful arguments also exist for the maintenance of its existence under *T. mentagrophytes* type VIII. This ensures taxonomic stability, highlighting the continued variation within the *T. mentagrophytes* complex, rather than designating as a new species²⁹.

The present study has also dealt with the *in-vitro* drug susceptibility to antifungal agents belonging to azole and allylamine groups and the resistance determinants exhibited by the recalcitrant dermatophytes. Itraconazole and fluconazole were tested against isolated dermatophytes, and itraconazole was found to be the most efficacious with 34.9 per cent sensitivity at the lowest concentration of 0.0625 mg/L of the antifungal agent, sensitivity of dermatophytes was poor for fluconazole. Terbinafine, an allylamine, presented 37.9 per cent sensitivity at the lowest concentration (0.0625 mg/L), and resistance was observed in 15.1 per cent of the isolates, with MIC being more than 32 mg/L. Additionally, terbinafine resistance (Wild type \leq 0.125 mg/L) according to the breakpoint described by EUCAST guidelines was observed in 16 (64%) isolates out of 25 isolates and among genotypically identified as *T. mentagrophytes*, nine (53%) isolates showed terbinafine resistance.

Resistance to terbinafine has been contributing to treatment failures and relapses, as seen in studies where nearly half of the patients experienced relapses^{30,31}. Terbinafine inhibits the enzyme squalene epoxidase, which converts squalene to 2,3-oxidosqualene, a critical step in ergosterol biosynthesis. This inhibition causes the accumulation of squalene, which is toxic to fungal cells. Alternatively, itraconazole inhibits the enzyme lanosterol 14- α -demethylase, which blocks the conversion of lanosterol to ergosterol, disrupting fungal cell membrane synthesis. Mutations in squalene epoxidase can reduce terbinafine binding by altering structure without disrupting its ergosterol synthesis role. Mutations in 14- α -demethylase are responsible for itraconazole resistance, though these are reported only in non-dermatophytes³².

Yamada *et al*³³ identified several point mutations leading to L393P, L393S, P397I, P397L, P397V, P415V, and H440T substitutions in *T. rubrum* isolates and *T. interdigitale* isolates exhibiting allylamine resistance. In the present study, point mutation was observed in five isolates at P397L, P415S and P415I, which is in accordance with the work conducted by Rudramurthy *et al*⁹ wherein out of 20 isolates, only seven isolates showed mutation in the squalene epoxidase gene. Among two of the *T. mentagrophytes* isolates with MIC >32 mg/L, only sample-29 isolate showed the presence of mutation at P397L position, and all the four isolates identified as *T. indotineae* (Sample-31, sample-32, sample-33 and sample-38) showed mutation at multiple amino acid sites. Sample-33 was the isolates with the

multiple mutation, which presented MIC of 32 mg/L concentration of all the antifungal agents.

Several studies report a lack of correlation between *in vitro* susceptibility and clinical outcome, especially in recalcitrant dermatophytosis^{34,35}. The high *in vitro* sensitivity of recalcitrant dermatophytes to terbinafine and itraconazole suggests that the perception of its inefficacy may be attributed to factors such as improper dosing, poor patient compliance, socioeconomic and hygiene issues, or the use of substandard brands. These elements can significantly impact the effectiveness of the treatment, leading to misconceptions about the drug's overall efficacy³⁶. Further, prolonged treatment with antifungal agents has been shown to provide better clinical outcomes irrespective of mutations in the squalene epoxidase gene³⁷. Additionally, virulence factor generated by the dermatophytes in response to the host also can contribute to the clinical resistance. In the present study, enzymatic and non-enzymatic virulence factors were observed: *T. mentagrophytes* complex and *N. gypsea* isolates produced keratinase (100%), lipase (87.76% and 69.23%), phospholipase (87.76% and 76.92%), protease (79.59% and 69.23%), gelatinase (79.59% and 100%), and melanin (77.55% and 76.92%). However, among *T. rubrum* isolates, none of isolates produced keratinase, lipase and protease was produced by one each and phospholipase was produced by two isolates. Gelatinase and melanin production was observed in 100 per cent of *T. rubrum* isolates, which agrees with many other studies¹⁴. Statistically significant ($P < 0.5$) number of antifungal resistant isolates produced proteases compared to those that were sensitive to the antifungal agents.

The study emphasises the need for continuous monitoring of antifungal resistance patterns, a better understanding of the genetic mechanisms underlying virulence and resistance. Such measures are crucial for developing effective treatment strategies and betterment of patient outcomes in the fight against recalcitrant dermatophyte infections.

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Conflicts of Interest: None.

Use of Artificial Intelligence (AI)-Assisted Technology for manuscript preparation: The authors confirm that there was no use of AI-assisted technology for assisting in the writing of the manuscript and no images were manipulated using AI.

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