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"A STUDY ON ANTIFUNGAL SUSCEPTIBILITY PATTERN, MOLECULAR PROFILING AND ITS SEQUENCING ANALYSIS OF TRICHOPHYTON RUBRUM ISOLATED FROM A TERTIARY CARE CENTRE".

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ABSTRACT

Introduction: *Trichophyton rubrum* is the most common aetiological agent of human dermatophytoses. These infections mainly occur in keratinised layers such as skin, hair and nails because the fungus uses keratin as a nutrient source. *T. rubrum* being the most common etiological agent isolated from clinically diagnosed lesions of cutaneous dermatophytosis. It has a significantly higher capacity of transmission than other anthropophilic dermatophytes.

Aim and Objective: To study the Antifungal Susceptibility Pattern , Molecular Profiling and its Sequencing Analysis of *Trichophyton rubrum* isolated from patients.

Material and Methods : This was a prospective study carried out in the Department of Microbiology. A total of 380 clinically suspected cases of superficial fungal infection were collected with proper informed consent. Dermatophytosis was confirmed in cases by direct microscopy and culture methods. Antifungal drug susceptibility profiles, MIC of isolates were 50 90 determined using the broth microdilution method according to the CLSI guidelines 2022. The DNA isolation was done using the Qiagen DNA extraction kit followed by the conventional PCR. The sequencing methods were used for the genetic analysis of virulence gene for the confirmation.

Results : In the present study out of the total of 380 clinically suspected cases of superficial fungal infection 39 isolates of *Trichophyton rubrum*were studied. The ratio of Male were more 32 (82.05%) as compared to the Female 7(17.94%). The maximum number of isolates was found in the Skin 36(92.30%) followed by the Nail 3 (7.69%). The ITZ was most sensitive toward *T. rubrum*

than KTZ, TBF, and FCZ. The molecular characterization for the detection of 18s gene was performed which was confirmed by the Sequencing Analysis.

Conclusion : The fungal genome's genetic diversity has led to an increase in antifungal resistance against *T. rubrum* during the past few decades. The gold standard method for identifying *T. rubrum* is molecular identification, which plays a significant role in the development of strategies for the prevention and treatment of fungal infections.

Keywords: Antifungal Susceptibility, *Trichophyton rubrum*, Molecular Profiling, DNA, PCR, Sequencing

INTRODUCTION

Dermatophytes are a group of closely related species that are keratinophilic and morphologically similar. They have the capacity to invade the keratinized tissue (skin, hair, and nails) of humans and other animals to produce an infection, dermatophytosis, commonly referred to as ringworm [1,2]. The universal occurrence of dermatomycosis as estimated by the World Health Organization is about to be 20% [3]. Ringworm is caused by the members of three genera *Microsporum*, *Trichophyton*, and *Epidermophyton*. These keratinophilic pathogenic organisms are also saprophytic in nature. *Microsporum* and *Trichophyton* are human and animal pathogens. *Epidermophyton* is only a human pathogen [1,2].These infections are not life-threatening, but they cause physical discomfort to the affected persons, which may even lead to a lower self-esteem.

The incidence of such infections is on the rise, especially in the immunocompromised patient groups including AIDS, diabetes mellitus, cancer and organ transplantation patients, etc. [2]. These are also associated with secondary bacterial infections that may lead to systemic skin infections [4,5].

Within the past 5 years, there has been a sudden, unexplained surge in dermatophytoses in India [6]. Dermatophytosis is usually regarded as an unimportant health issue for human and animals since a wide range of antifungal drugs is available. In recent times, however, epidemics by several dermatophyte species have been reported around the world with considerable virulence and resistance to commonly applied antifungals [3]. *T. rubrum* is the most frequent causative agent of human dermatophytosis that can escape or suppress the host immune system during the infection process and, unlike other fungi, can cause infections in healthy, immune-competent people. The microorganism is specialized to infect humans, but there are some studies reporting animal infections.

This epidemic has been characterized by recurrent, recalcitrant infections characterized by unusual clinical patterns including erythrodermic dermatophytoses, pseudoimbricata patterns, and scalp involvement in adults [7]. These patterns of dermatophyte infections have been observed even in patients who have been compliant with standard antifungal therapies. However, there are very few studies documenting the present clinico epidemiological patterns of dermatophytoses in India. More importantly, there are even fewer studies outlining antifungal susceptibility of common dermatophyte species in India. The paucity of epidemiological data, coupled with so far undetermined breakpoint minimum inhibitory concentration (MIC) values of antifungals against dermatophytes adds further to the difficulty in managing the current dermatophyte epidemic.

Trichophyton infections are extremely difficult to diagnose and can take months to treat. Thus, there has been a lot of interest in quick diagnostic tests that use exact molecular techniques [6]. Another significant issue with clinical therapy is the growing resistance to antifungal medications. When topical therapy is insufficient to treat severe dermatophyte infections, such as those affecting the nails, scalp, or feet, systemic antifungal medications are recommended. It has been demonstrated, therefore, that treatment options are limited due to the low success rate of therapies and the elimination of antifungal medication classes due to drug resistance. Novel insights into the genetic

variables controlling this resistance to antifungals and the processes involved in fungal disease offer a basis for innovative treatment approaches.

Molecular sequencing studies revealed that *T. rubrum* has higher clonal conserved gene content, low variability, and recombination [8]. Moreover, low virulence is one of the significant factors for enhancing the global transmission of *T. rubrum* [9]. Conventional methods including KOH microscopy and culture are the first line of strategy to diagnose *T. rubrum*. The culture method offers microbial colonies which are further utilized for more precise identification and characterization by employing molecular analysis using PCR and sequencing tools [10].

The identification of *T. rubrum* by means of conventional laboratory methods may not always be easy or straightforward, since *T. rubrum* exhibits substantial phenotypic variability. Contrastingly, a high degree of homogeneity of the *T. rubrum* genome, as revealed by using several anonymous molecular markers, significantly impedes discrimination at the strain level [11,12]. Yet, as new molecular typing methods are becoming increasingly available, species determination and strain typing are still being improved.

To better understand and identify antifungal drug response patterns in *T. rubrum* for the future management of this disease, the current study was conducted to examine the antifungal susceptibility pattern, molecular profiling, and sequencing analysis of *Trichophyton rubrum* isolates.

MATERIAL AND METHODS

This was a prospective study carried out in the Department of Microbiology for a period of 1 year i.e, August 2022 to August 2023. A total of 380 clinically suspected cases of superficial fungal infection were collected with proper informed consent.

Inclusion criteria: The study included subjects of all age groups and any gender with clinically diagnosed dermatophytosis were studied.

Exclusion criteria: Any subjects with the history of use of antifungal agents for 4 weeks were excluded from the study.

PHENOTYPIC METHOD:

Identification and Sample processing

The Clinical Samples isolated from skin and nails were subjected to direct microscopic examination and conventional culture method via scalping using 10% KOH and 20% KOH for skin and nails respectively. *T. rubrum* was cultured in Saboured dextrose agar with chloramphenicol and cyclohexamide (Hi Media) at 25-30 °C for 2-4 weeks and follow-up at intervals during incubation to examine the progress of colony formation. Further, microscopic slide culture or microculture was prepared to morphologically characterize this dermatophyte using light microscopy according to the CLSI guidelines 2017 [13].

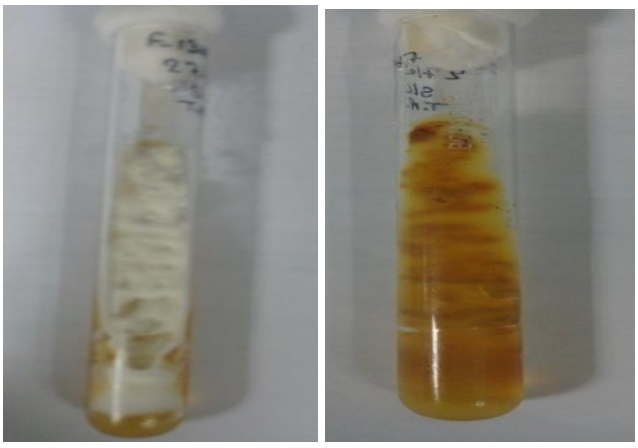


Figure No. 1: *T. rubum* colony appearance: White to cream

Antifungal Susceptibility Pattern of *T. rubrum*

T. rubrum's in vitro antifungal susceptibility pattern was ascertained using the broth microdilution assay (BMA) technique in accordance with the standard guideline (CLSI-M38-3rd edition, 2022) [13].

The references strain of *T. rubrum* (ATCC 10218) was uses as quality control. The antifungal potential reagent grade powder of Fluconazole and Itraconazole (Metro Chem API Pvt. Hyderabad, India), Ketoconazole (Arti drugs Ltd. Maharashtra, India), and Terbinafine (Shreeji Pharma International, Sarabhi, Vadodara, Gujarat, India) were used. All drugs were dissolved in RPMI-1640 medium supplemented with L-glutamine and without sodium bicarbonate (pH 7.0, adjusted with 0.165 M of morpholinepropanesulfonic acid) along with 1N NaOH (Hi Media). The final concentrations of antifungal agents were 0.5 to 64 µg/ml, for fluconazole, whereas 0.0078 µg/ml to 128 µg/ml for Itraconazole, Ketoconazole, and Terbinafine. Spore suspensions of *T. rubrum* were prepared as protocol previously described by [14].

Briefly, cultures of fungal isolates (7-8 days old) were grown on SDA slants at 25°C and used to prepare inoculums. To obtain the final inoculums (2-6×103 CFU/mL) cell suspension was diluted in RPMI-1640 medium. Further, 100 µl cell suspension was seeded in each well of 96 well plates and incubated with different antifungal stocks. After thorough mixing plates were incubated at 35 °C for 4-5 days.

The point at which dermatophytes were inhibited by the lowest concentration of drugs relative to the control was designated as the minimum inhibitory concentration (MIC) of the antifungal agent. Furthermore, against T. rubrum, MIC50 and MIC90 values were found. To eliminate statistical biases, duplicate runs of every experiment were conducted.

GENOTYPIC METHOD:

Molecular Identification of *T. rubrum*

The DNA was isolated using the Qiamp DNA Blood Mini Kit (QIAGEN, Germany) as per the manufactures guidelines. . The DNA was eluted in 60 µl elution buffer and preserve at -20 °C till PCR analysis. For amplification of the target gene, PCR was carried out in a 50 µL reaction mixture. The 18SrDNA region of fungal genomic DNA was amplified by using fungal primer sequences specific for DH1 were used . The primers were purchased from "**Saha gene"** and was reconstituted with sterile double distilled water based on the manufacturer's instruction.

Gene	**Primer sequence**	**Length (bp)**
18SrDNA	Forward- 5'-TGC ACT GGT CCG GCT GGG-3'	180bp
	Reverse 5'-CGG CGG TCC TAG AAA CCA AC-3'	

Table No. 1 : Primers for Gene Polymorphism [15]

The PCR cycling conditions used were one initial cycle of 3 min at 94°C, followed by 35 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 58 °C, and 1 min of extension at 72 °C, with a final extension of 7 min at 72 °C. The resulting PCR product of approximately 160-180 bp was subjected to 1 % agarose gel electrophoresis and visualized by Gel Doc™ EZ Gel Documentation System (Bio-Rad Laboratories Inc., Hercules, CA, USA). A 100-bp molecular marker (Fermentas Life Sciences, Waltham, MA, USA) was used to evaluate the PCR product of the sample [15].

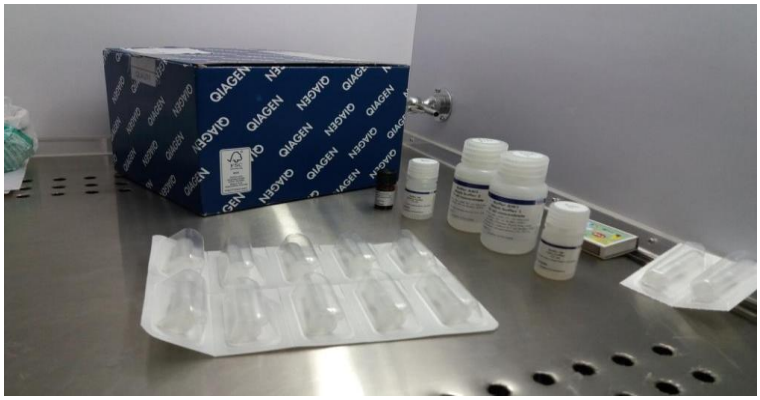


Figure No. 2: The DNA Extraction Qiagen kit and the Reagents used for the DNA Extraction

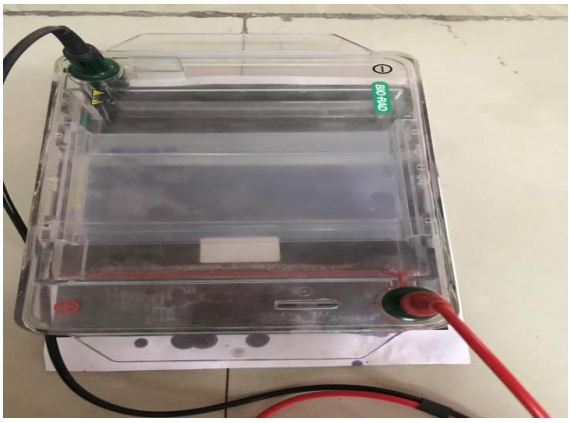


Fig No.2: Gel Electrophoresis for the DNA Extraction

The Sequencing Analysis of *T. rubrum*

After being packaged, the PCR product was sent to Applied Biosystems in Bangalore for sequencing analysis. Sanger sequencing of the ITS region was used to confirm the molecular identity. In order to identify similarities and differences between nucleotide sequences, the sample's forward and reverse sequences were run using the BioEdit software version 7.0.5. Manual revision was then performed to increase the accuracy of the alignment. For the purpose of identifying and comparing fungi, the sequence data was integrated into the Centraalbureau Voor Schimmel cultures (CBS) and the NCBI's Basic Local Alignment Search Tool (BLASTN).

Statistical analysis

The statistical analysis was done by *t*-test using SPSS 20 software in order to find the independence of the variables or whether they had similarities in their MIC values with $P < 0.005$.

RESULTS

In the current study a total of 380 clinically suspected cases of superficial fungal infection were included out of which 39 isolates of *Trichophyton rubrum* were observed after clinical identification with conventional diagnostic approaches such as KOH microscopy and culture method. *T. rubrum* isolates appeared as flat to slightly raised white to cream, pseudo-like to velvety [Figure 1]with a yellow-brown to wine- red pigment, which was further confirmed microscopically.

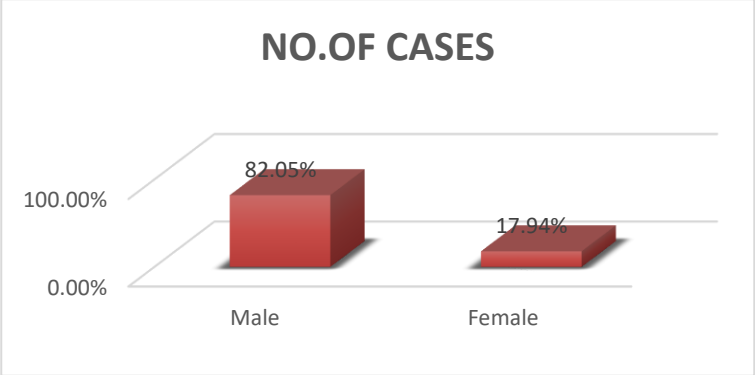
Type of Isolates	Total Number of Isolates
Clinical Isolates	380
Trichophyton rubrum	39

Table No. 2: The total number of Isolates

The ratio of Male were more 32 (82.05%) as compared to the Female 7 (17.94%). The maximum number of isolates was found in the Skin 36 (92.30%) followed by the Nail 3 (7.69%). The ITZ was most sensitive toward *T. rubrum* than KTZ, TBF, and FCZ. The molecular characterization for the detection of 18SrDNA gene was performed which was confirmed by the Sequencing Analysis.

Gender	Total no. of Cases studies	Percentage
Male	32	82.05 %
Female	7	17.94%
Total	**39**	

Table No. 3 : Genderwise distribution of the*Trichophyton rubrum* cases.



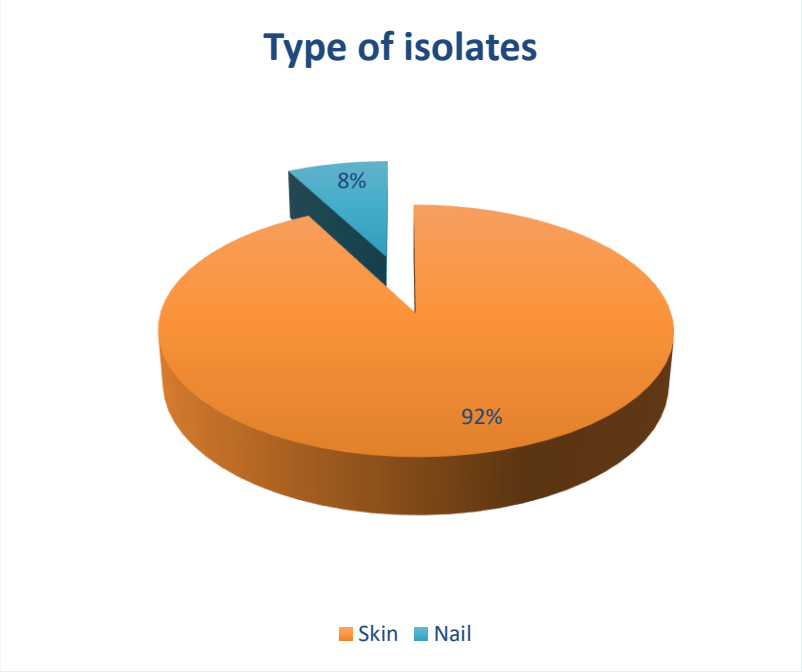
Graph No. 1: Graphical representation of Genderwise distribution

S.No.	Age (in years)	No. of Cases	Percentage
1.	0- 10	-	-
2.	11-20	3	7.69%
3.	21-30	18	46.15%
4.	31-40	11	28.20%
5.	41-50	2	5.12%
6.	51-60	2	5.12%
7.	≥61	3	7.69%

Table No.4 : Agewise distribution of *Trichophyton rubrum* patients from the study

Type of Isolate	Number of Isolate	Percentage
Skin	36	92.30%
Nail	3	7.69%

Table No. 5: The Type of Isolate



Graph No. 2: Graphical representation of the Type of Isolates

In our study, the antifungal susceptibility trend of various antifungal agents was evaluated against *T. rubrum* as depicted below in Table No. 6.

Antibiotics	Fungal species	MIC range (µg/ml)
Itraconazole	*T. rubrum*	0.0157-0.25
Ketoconazole		0.0157-0.5
Terbinafine		0.0312-16
Fluconazole		2-64

Table No. 6: In vitro Antifungal susceptibility of *T. rubrum* against different antifungal Drugs

From the Table No. 6 it was observed that the Itraconazole showed the highest antifungal potential (MIC range 0.0157-0.25 µg/ml) against *T. rubum* followed by Ketoconazole (MIC range 0.0157-0.5 µg/ml), terbinafine (MIC range 0.0312-16 µg/ml), fluconazole (MIC range 2-64 µg/ml) respectively. Our finding demonstrated that Itraconazole might be used for the management of *T. rubrum* infection. Furthermore, our results confined that there was no significant difference recorded between susceptibility patterns of antifungal agents within studies regions.

The Dermatophyte-specific PCR and Sequencing of *T. rubrum* genome

T. rubrum was identified by Conventional PCR using 18S ribosomal DNA region-specific primers which resulted in 180 bp of undigested PCR product conferring the selective and specific amplification of the 18S rDNA region of *T. rubrum*.

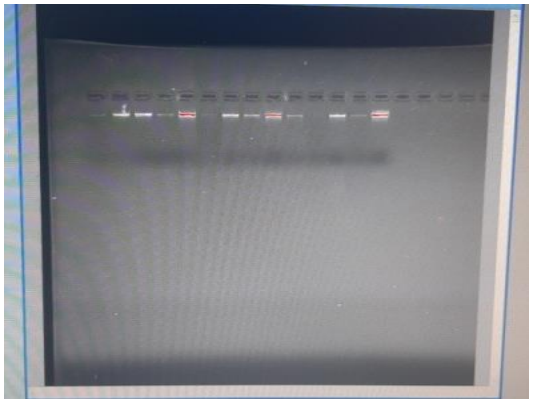


Figure No. 4: The DNA isolated

L1,L2,L3,L4,L5,L6,L7, L, L8,L9,L10,L11,L12

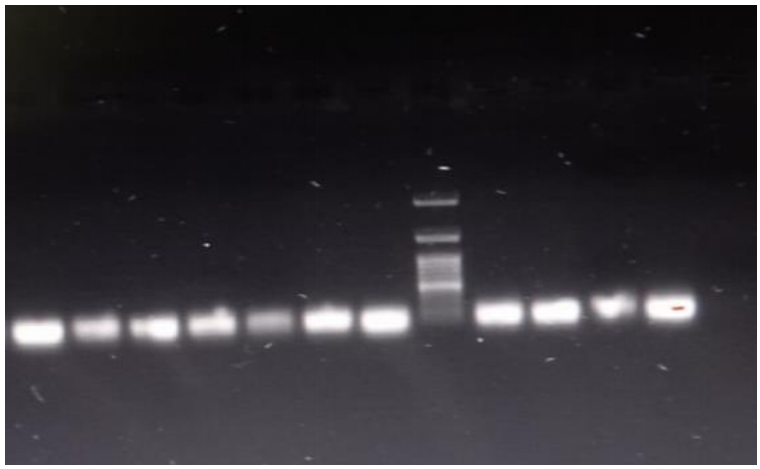


Figure No. 5: The DNA Amplification outcome of 18S rDNA region of *T. rubrum* using Agarose gel Electrophoresis. Lane L1-L7 and L8-L10 was 18S rDNA region of *T. rubrum* amplified 180 bp region using dermatophyte specific primers. Lane L corresponds to the 100 bp DNA Ladder. L 11 is the positive control for *T. rubrum* and Lane L12 negative controls DNA visualized by Gel documentation system

Further, *T. rubrum* species was validated through sequencing analysis using the NCBI Gen Bank database (GBD) by BLASTn. The sequence of representative fungal species *T. rubrum* was 100% identical when compared to the gene sequence of control *T. rubrum* strain ATCC 10218.

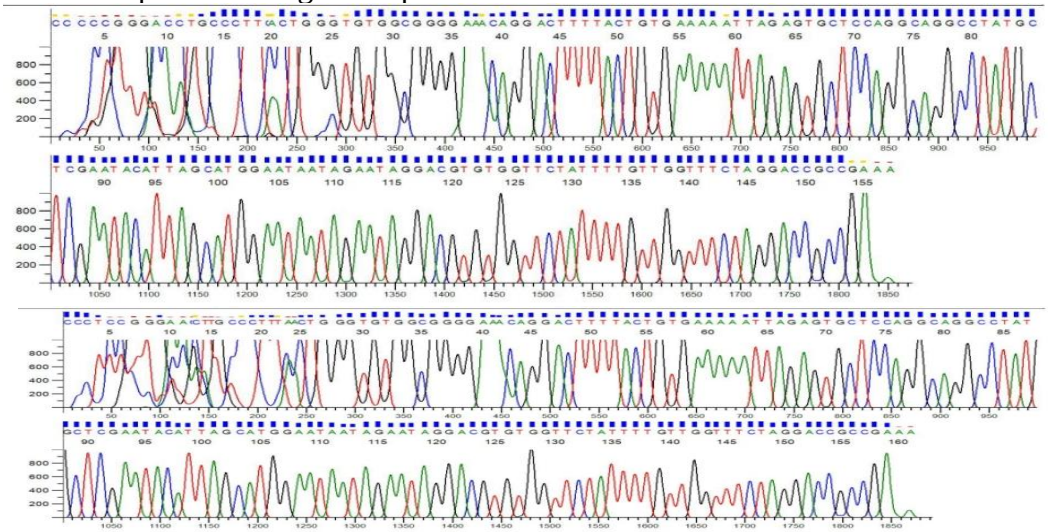


Figure No. 6: Sequencing analysis of *T. rubrum* isolate displaying 100% similarity and coverage to control strain.

>LC633537.1 Trichophyton rubrum R.H.I gene for hypothetical protein, partial cds
ACTGGATCTACCAGTGGTGTTATCCAGGGTATCCAGTGGTGTACCGACCATGCTGGTAG
AAATGGTTTGAGAGGCAAGGCAGCCATGAACCTGAGCCTGGGTATCCGTGGCTCAACT
GTTTTCAACCGCGCTGCAGAAGCAGCCCAACAGTCCGGTATTTTTCTAGCTGTGGCGGC
AGGGAATGA

DISCUSSION

Over the past few years, it has been documented that dermatophyte infections have increased by many folds in India [2]. *Trichophyton rubrum* is a dermatophytic fungus in the phylum Ascomycota. It is an exclusively clonal, [11] anthropophilic saprotroph that colonizes the upper layers of dead skin, and is the most common cause of athlete's foot, fungal infection of nail, jock itch, and ringworm worldwide[16].Typical isolates of *T. rubrum* are white and cottony on the surface. The colony underside is usually red, although some isolates appear more yellowish and others more brownish. . *Trichophyton rubrum* grows slowly in culture with sparse production of teardrop or peg-shaped microconidia laterally on fertile hyphae. Macroconidia, when present, are smooth-walled and narrowly club-shaped, although most isolates lack macroconidia [17].

Moreover, there is a change in the disease presentation, severity, treatment response, and relapse rate.

The climatic conditions (hot and humid environment pertaining to tropical and sub-tropical regions) in India are favourable for the development of superficial fungal infections [16]. The other factors that aid these infections include unhygienic living standards, especially prevalent among the low socio-economic strata, and a high population density especially seen in communities like the ones around construction sites.

Millions of cutaneous fungal infections are observed in humans annually in which dermatophytosis ranks the first with 10-20% of the population been affected by dermatophytes worldwide [17].

In the present study the maximum number of isolates was found in Males 32 (82.05%) as compared to the Females 7 (17.94%). This finding was in accordance with Dubas et al., [18] where the ratio of males (64%) was comparatively more than females (36%). There was Similar observation been made by Gamage et al [19] and Shital et al [20] where the ratio of males was comparatively more than females . This may be due to the general characteristic of this age group as they are more involved in outdoor activities involving physical labour [21,22]. The higher prevalence amongst males may also prove to be an occupational hazard.

In present study we observed that the maximum number of cases for *T. rubrum* was found in the age group of 21-30 years of age followed by 31-40 years and the least in the age group below 20 years of age. This study was similar to study performed by Kak et al.,[23] . In the present study, skin infections were found to be the commonest clinical presentation (90.3%) followed by nail infection (9.6%). This study was Similar observation by Patel P et al., and Kannan et al., in their study who reported dermatophytic skin infection to be more common compared to dermatophytic nail and hair infections [24,25].

Molecular techniques offer an advanced technique to identify dermatophytes and are preferred over conventional approaches. In the present study the detection of 18s gene of *T.rubrum* was done. Therefore, the implementation of molecular analysis tools for genomic level identification is very important to distinguish between strain and species at the gene level.

Over the last few decades, the most commonly studied antifungal drugs are ITZ, FCZ, KTZ, and TBF. Response of antifungal agents plays a vital role in the management of spermatophytes. In this study in vitro antifungal sensitivity of different antifungal agents towards *T. rubrum* infection was recorded. We noted that Itraconazole (ITZ) showed the highest sensitivity against *T. rubrum* , while the sensitivity of Ketoconazole (KTZ), terbinafine (TBF), and fluconazole (FCZ) were recorded lower. Afshari et al.[26] also reported the highest sensitivity of ITZ against *T. rubrum* along with E. floccosum. Instead, a recent finding from china found TBF as a most sensitive antifungal drug for *T. rubrum* with MIC value (GM = 0.00688 lg/ml, MIC50 = 0.008 lg/ml, MIC90 = 0.015 lg/ml) (Jiang

et al., 2021)[27]. However, data demonstrated that *T. rubrum* isolated from the different geographical areas of India was resistant to TBF (Ebert et al., 2020) [28].

From these observations, we can infer that antifungal susceptibilities vary widely with geographical attributes even with identical testing methods. There is however an evident trend of rising MIC-90 to terbinafine in India though a clear statement about resistance to terbinafine cannot be made due to lack of CLSI guidelines defining the drug breakpoints. This emphasizes the need for several such studies from different regions in India along with *in vivo* correlation to define the MIC breakpoints. Recent Indian reports of mutation in *squalene epoxidase* gene in *Trichophyton* isolates support our observation of high MIC-90 to terbinafine.

Molecular techniques offer an advanced technique to identify dermatophytes and are preferred over conventional approaches. In the present study the detection of 18s gene of *T.rubrum* was done. Therefore, the implementation of molecular analysis tools for genomic level identification is very important to distinguish between strain and species at the gene level.

These days, PCR-based molecular methods, which may diagnose superficial fungal infections, have improved sensitivity, specificity, and quick findings. In this work, the isolated *T. rubrum* strain from that specific location of India was identified using a PCR-based method, which was then further confirmed using a sequencing tool by comparing with a control. DNA sequences are very useful for this purpose and permit an accurate identification. The present study might be helpful for the management of *T. rubrum*-associated fungal infection in that particular region of India. Poor hygiene among low socio economic group, environment and climatic conditions plays a vital role in causing the infection. Early diagnosis and identification is the key for prevention and treatment of dermatophytosis among such population [29].

Conclusion

Given the hot and muggy weather in India, dermatophytosis poses a serious health risk. As a result, regular monitoring is required, as is raising awareness of the appropriate preventive measures in such areas. The antifungal medications that were most effective against T. *rubrum* were Itraconazole and Ketoconazole combined. may enable medical professionals to manage this illness and enhance treatment outcomes by deliberately monitoring aggression.

Declarations:

Conflicts of interest: There is no any conflict of interest associated with this study

Consent to participate: We have consent to participate.

Consent for publication: We have consent for the publication of this paper.

Authors' contributions: All the authors equally contributed the work.

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