Analysis of ERG 11 Expression in Clinical Isolates of Dermatophytes in Patients with Resistant Tinea Infection
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ABSTRACT

Background: Dermatophytes are keratinophilic groups of microorganisms which invade keratinized tissue. Isolate and identify the dermatophyte species from samples collected from patients suffering from onychomycosis, tinea corporis and tinea cruris; and perform in vitro microbroth antifungal susceptibility testing. Further, the expression of Ergosterol 11 (ERG 11) amongst the isolates from responders and non-responders to antifungal treatment was studied by Real-time PCR.

Methods: A total of 120 dermatophytosis cases attending a dermatological clinic in a tertiary care hospital were included in the study. Microscopy of KOH mount was positive in 90 isolates while 60 were culture positive.

Results: The most common dermatophytes implicated were Trichophyton mentagrophytes (75%) followed by Trichophyton rubrum (25%) which were molecularly confirmed by PCR using the species-specific primer. Higher MIC was detected for fluconazole (10 %), itraconazole (3.33 %), terbinafine (10 %) and griseofulvin (5 %), for T. mentagrophytes and T. rubrum, while all strains showed lower MIC for voriconazole and luliconazole.

Conclusion: The study observed a predominance of T. mentagrophytes causing chronic dermatophytosis. The rising MIC to terbinafine and griseofulvin among the isolates raises a concern for effective management. The real-time PCR analysis of ERG 11 expression in certain isolates demonstrated up-regulation in patients not responding to treatment as compared to responders, the impending failure of azoles as a line of treatment in dermatophytosis.

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Introduction

Dermatophytes, a group of keratinophilic filamentous fungi thriving on the keratin substrate, are the etiological agents responsible for causing superficial fungal infection in human and animals with an estimated global prevalence of approximately 20 percent as per the World Health Organization (WHO) report. Predominant in the tropical and subtropical countries; especially in the developing countries like India, the hot and humid climate is favorable to the acquisition and maintenance of the disease.1 Routine procedures for dermatophyte species identification is based on the conventional phenotypic method of microscopy and culture. Morphological and physiological characteristics can frequently vary, often influenced by temperature variation, and type of medium, further hampering strain identification. In the last few years genotypic approaches have proven to be useful for addressing taxonomic complexities in dermatophytes; moreover, genotypic detection is considered more stable and precise than phenotypic characteristics.2-5

In the past few years, chronic dermatophytosis has become a public health problem. Recalcitrant dermatophytosis encompasses relapse, recurrence or re-infection & persistence of infection. Systemic antifungals are used for treating dermatophytosis as monotherapy or in combination, and azoles are still used as the first line of management in several cases. Azoles inhibit the fungal cytochrome P-450 enzyme lanosterol 14-α-demethylase (Cyp51) encoded by ERG 11 gene, being effective against several fungi including the common dermatophytes like Trichophyton rubrum, Trichophyton mentagrophytes, and Epidermophyton floccosum as documented by Garvey et al.6 Studies have reported increasing minimal inhibitory concentration (MIC) values to fluconazole, terbinafine and griseofulvin amongst dermatophytes leading to clinical failure. The potential loss of efficacy of azoles has prompted many researchers to make concerted efforts to discover new drugs that might block fungal growth at different metabolic sites.7,8 Hence this study was aimed to co-relate the MIC values with the expression of ERG 11 genes by real-time PCR for fluconazole and itraconazole and to correlate the expression with MIC and clinical cure.

Materials and Methods

The present study was conducted on a total of 120 samples from clinically diagnosed dermatophytosis patients, subjected to culture from skin and nail samples of patients attending dermatology OPD of a tertiary care hospital, Delhi from January 2016 to December 2018. A portion of each clinical specimen was suspended in a drop of 10% & 40% potassium hydroxide (KOH) for processing skin & nail respectively. KOH wet mount slides were viewed under a light microscope under 40X magnification. A portion of the sample was cultured on Sabouraud’s dextrose agar (Hi-media, Mumbai) with antibiotics with chloramphenicol (0.05 g/l), gentamicin (20 mg/l) and cyclohexamide (0.5 g/l). All inoculated tubes were incubated at 25°C for optimal growth. After growth, the etiological agent was confirmed by the characteristic morphology of the colony and by
studying the microscopic appearance of the fungus on Lacto Phenol Cotton Blue (LPCB) mount and Urease test. The molecular confirmation of isolates was done by using species-specific primers of T. mentagrophytes and T. rubrum.\textsuperscript{9}

**DNA extraction and PCR**

DNA was extracted from the cultures grown on SDA by using the commercially available DNA extraction kit (HiYield Genomic DNA Kit, RBC, Taiwan). On the basis of alignment of sequences of internal transcribed spacer region ITS 1 and 2 in the NCBI nucleotide database, a primer of T. rubrum forward (203bp) GACCGACGTTCCATCAGGGGT and reverse TCAGACTGACAGCTCTTCAGAG and T. mentagrophytes (130 bp) forward CAAACGTCCGTCAGGGTGAGC and reverse TAGCCACTAAAGAGAGGCTCGC were used for amplification.\textsuperscript{10,11} Each tube contained a total volume of 25 μl which included 2.5 μl buffer (10X), 5 μl of Q-buffer, 0.5 μl dNTPs (200 μM), MgCl\textsubscript{2} 0.5 μl (1.5 mM), 0.15 μl Taq polymerase, 1 μl of each primer, forward and reverse (10 μM), 5 μl of the extracted DNA and nuclease-free water to make up the volume. All PCR reagents were from Taq PCR Core Kit (Fisher Scientific – Qiagen, Germany). Amplification was performed in a Mastercycler personal (Eppendorf, Hamburg, Germany). Initial denaturation was performed at 94°C for 10 min which was followed by 35 amplification cycles of 30 s at 95°C and 45 s at 65°C and 30 s at 72°C, and final extension of 10 min at 72°C. The amplified PCR products were analyzed by electrophoresis on 1.5% agarose gel, stained with Ethidium bromide at 125 V and 15 mAh current in a 10 slot apparatus for 30 min. Molecular marker of 100 bp was used to determine the size of the amplicons.\textsuperscript{12,13} Standard ATCC control strains were used as positive controls (T. mentagrophytes ATCC. 28185).\textsuperscript{14}

Purification of PCR products and DNA sequencing analysis was performed by comparison of the nucleotides with dermatophytes reference nucleotide sequence obtained from gene bank database (site http://www.ncbi.nih.gov/gene bank). The isolates were identified as T. mentagrophytes and T. rubrum with 99% similarity. The representative sequence obtained was submitted to gene bank database; T. mentagrophytes accession number are MH644185, MH745112, MH778308, and T. rubrum accession number are MH497367, MH497368, MH497369.

**RNA isolation**

Total RNA from culture isolates obtained from skin and nail were extracted by TRIzol \textsuperscript{TM} Reagent (Invitrogen Bio services India Pvt. Ltd) method. Isolates were mixed in TRIzol \textsuperscript{TM} Reagent, 1 ml of RNA express reagent was added in the sample, total lysate cells were obtained, which was further mixed by micropipette to form a homogeneous lysate and incubated for 5 min at room temperature. 200μl of chloroform per ml of RNA express reagent was added and centrifuged at 12,000 rpm for 15 min at 4°C to obtain a colorless upper aqueous phase containing RNA which was separated in another 1.5 ml Eppendorf tube. RNA was washed by adding 500 μl of Isopropanol. The Eppendorf tube was centrifuged at 12,000 rpm for 10 min at 4°C and supernatant was discarded.1 ml of 75% ethanol was added to the pellet and vortexed. This was followed by centrifugation at 10,500 rpm for 5 min at 4°C. The supernatant was discarded, and the pellet was suspended in RNase free water, which was incubated at 55-60°C for 15 min. The isolated RNA was stored at -80°C immediately. The purity and integrity of the RNA samples were confirmed by agarose gel electrophoresis. Nano-drop
determined the concentration and purity of the samples and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as an endogenous control.

**cDNA Preparation:** Extracted RNA was used as a control. The PCR program was 95°C for 10 min, followed by 40 cycles of 95°C for 10s and the annealing/extension at 60°C for 20s and 72°C for kit. Total RNA (2-5 µg) from each sample was reverse transcribed into cDNA.

**Gene expression of Ergosterol 11 in T. rubrum & T. mentagrophytes by Quantitative Real-Time PCR (qRT-PCR):**

To quantify expression of Ergosterol 11 (ERG 11) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), qRT-PCR was performed using the Light Cycler® 480 Instrument (Roche Diagnostics, Germany). Thirty isolates were chosen and categorized into two groups (15 isolates in each group) as clinical non-responders to antifungal drugs and responders to antifungal treatment.

Real-Time PCR reaction mixtures contained: Light Cycler® 480 SYBR Green I Master Mix, Nuclease-free water (Roche Diagnostics, Germany), cDNA and primers. Primers are ERG 11 forward 5’-CACTTCCTTGCCCTGTAGAGATC-3’, ERG 11 Reverse 5’-GGAGTTTTCAATGTCAGCAAGGTTT-3’, GAPDH Forward 5’-ACGGCTTCTTGAGAAGTCATG-3’ and GAPDH Reverse 5’-ATGTATTCGCGGATTTGCTGTCT-3’.

The final volume of each reaction was 20µl; the reaction mixtures contained 2-5µl of c-DNA, 20 µl of SYBER Green Universal Master Mix, and 160 nM of each primer, and the volume was brought to 20 µl with nuclease-free water. The Real Time PCR condition had been standardized according to the melting temperature (Tm) of the primers. The Ergosterol-11 is the gene of interest and GAPDH, which served as an endogenous control.

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

Antifungal susceptibility testing was performed according to the Clinical Laboratory Standard Institute (CLSI) 38 A2 guidelines suggested for molds. *T. mentagrophytes ATCC 28185* was used as quality control. RPMI 1640 medium and MOPS (4-Morpholine propane sulfonic acid) (Sigma Aldrich, USA), pH 7.0 was used as a medium for suspension of the isolates. The antifungal drugs tested were fluconazole, itraconazole, terbinafine, griseofulvin, luliconazole and voriconazole (Sigma Aldrich, USA). The initial inoculum suspension of the isolates (i.e., 1 - 3 x 10^6 cells/ml) was prepared using the spectrophotometer to match the optical density of 80 % transmittance at 530 nm wavelength. The final concentration of the inoculum (1 - 3 x 10^3 cells/ml) is prepared in 1: 50 dilution in RPMI. The test assay which was performed in 96 U bottom microtitre plate was incubated for 96 hours at 35°C in BOD incubator.

Statistical analysis was done using Statistical Package for Social Sciences package (SPSS; Inc.,...
Chicago, IL, USA; version 20.0). The analysis comprised of calculating means and proportions. The independent sample t-test was used to test the statistical significance of the data.

**Study Design:** Cross Sectional Analytical Study.

**Sample Size:** 60 confirmed cases.

Based on this data, the sample size of 60 has been found to be statistically appropriate for this study (sample size calculated using the standard formula $Z^2P(1-P)/d^2$ for a prevalence study, where $Z = Z$ statistic for a level of confidence (the value of $Z$ is set at 1.96), $P = expected\ prevalence\ or\ proportion (in\ proportion\ of\ one;\ if\ 45\%,\ P = 0.45)$, and $d = precision\ (in\ proportion\ of\ one;\ if\ 125\%,\ d = 0.125)$).

$$n = \frac{Z^2 \alpha / 2 \times P (1-P)}{d^2} = \frac{1.96^2 \times 0.45 \times 0.55}{0.125^2} = 60.8$$

$P = 45\% i.e. 0.45$

$1-P = 55\% i.e. 0.55$

$d = 0.45 \times 0.25 / 100 = 0.125$

so we considering P rate of Trichophyton rubrum in dermatophytosis confirmed cases is 45% with 25% relative precision either the site with 95% confidence level is 60.8 i.e. total 60 cases is taken.

**Inclusion criteria:**

1. Patients between 1 month to 75 years with clinically suspected superficial fungal infection in nail, skin and hair.
2. Culture Positive cases of Trichophyton rubrum isolated from nail, hair & skin of these patients.
3. Genomic confirmations by PCR ITS 1, 4 primers of T. rubrum.

**Exclusion criteria:**

1. Patients with any systemic disease or autoimmune disorder, immunodeficiency, malignant disease or any immunosuppressive treatment.
2. Pregnant and lactating women.

**Source:** The present study was carried out in the Department of Microbiology and Dermatology UCMS and GTBH, New Delhi. Ethical clearance was obtained from UCMS &GTB Hospital.

**Results**

A total of 120 samples were collected which included 25 from onychomycosis patients and 95 skin scrapings from Tinea corporis and cruris patients. The mean age of patients was $32.5 \pm 11.32$ year (with the range from 16 to 60 years). The duration of dermatophytic infection ranged from 3 months to 10 years ($9.8$ months $\pm 5.45$ months). Out of 120 samples, 90 were found to be KOH positive, of which 60 samples were culture positive. The clinical profile of patients with culture-positive isolates (60) varied from those with first dermatophytic infection (26), those with no clinical cure despite antifungal therapy (30) and those with relapse (4).

Of the 60 isolates, 15 isolates were identified as *Trichophyton rubrum* and 45 isolates as *Trichophyton mentagrophytes* on phenotypic mycological assessment. *T. rubrum* was the predominant pathogen (66.67%) isolated from nail samples than from skin samples of Tinea corporis & Tinea pedis (20 %). *T. mentagrophytes* was majorly isolated from T. corporis and T. cruris (44.44 %) followed by T. faciae (13.33 %), T. pedis (11.11 %) and onychomycosis (4.44 %).

All isolates were also subjected to conventional PCR using the species-specific primer of their ITS region for further confirmation, which showed 100% agreement as in Figure A & B.
On antifungal susceptibility testing of the 60 isolates, the minimum inhibitory concentration (MIC) range for *T. rubrum* and *T. mentagrophytes* showed a maximum sensitivity of *T. rubrum* isolates to fluconazole (<64 µg/ml (18.33%); itraconazole (100%) at <1 µg/ml and terbinafine (21.6%) at <1 µg/ml whereas the sensitivity to *T. mentagrophytes* isolates was 71.6% to fluconazole; 71.6% to itraconazole, and 68.3% to terbinafine as depicted in Table I. High MIC for fluconazole (at >64 µg/ml) was detected in 10% isolates; for itraconazole (at >1 µg/ml) in 3.33%; terbinafine (at >1 µg/ml) in 10% and for griseofulvin (at >1 µg /ml) in 5% for both *T. rubrum* and *T. mentagrophytes* complex. Low MIC (100%) was observed for luliconazole and voriconazole. The mean and standard deviation of MICs of 60 isolates of dermatophyte was lowest for luliconazole, voriconazole, griseofulvin, itraconazole and terbinafine and highest for fluconazole in both *T. rubrum* and *T. mentagrophytes* isolates as shown in Table I. Most of the patients with the history of recurrence exhibited high MIC for either one or all the three drugs tested.

**Real-time PCR for Ergosterol-**

Real-time PCR was performed for 30 isolates categorized as 15 nonresponders and 15 responders. Both the groups were assessed for ERG 11 expression as fold change (by calculating $2^{-\Delta\Delta C_t}$ value). The Ct value of these ERG 11 genes was normalized by Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). The fold change in the level of expression of ERG 11 genes using Real-
Time PCR data and by applying \(2^{-\Delta\Delta C_T}\) method was 18.18-fold change and up-regulated between the two groups as depicted in Table II and Figure C. It was also seen that the level of ERG 11 expression was significantly different between the responders and the non-responder groups (\(t = 5.617, p<0.001\)) as shown in Table III.

**Figure-2:** Amplification curve peak of Ergosterol 11, GAPDH and Negative Control by Real-Time PCR.

**Discussion**

Chronic dermatophytosis has emerged as a significant problem in India with several cases of reinfection and relapse and failure to the treatment being reported across the country. The present study was conducted in patients with dermatophytosis, attending the dermatology OPD of a tertiary care hospital in East Delhi, India. The geographical condition of this part of our country has provided a favorable environment for the survival of the dermatophytes and adaptation to the human host for several decades.\(^1^7\) Besides the conducive environment, other factors that support the survival of the fungal agent, include unhygienic living condition, high population density, application of steroids, incomplete treatment and probable drug resistance.\(^1^8\)

As documented in other studies, males appeared to be more exposed to acquire dermatophytic infection (58.3 %) as compared to females (41.7 %) which is in accordance with the other researchers worldwide.\(^1^9\) Interestingly we found *T. mentagrophytes* complex was more prevalent than *T. rubrum* causing dermatophytosis in Delhi, similar to other studies.\(^2^0\) Out of 60 patients, 43.3% of cases were naive cases presenting with the first episode of dermatophytic infection, 50% of cases were previously on antifungals for 1-2 yrs and 6.6% cases categorized as relapse. The MIC range for *T. mentagrophytes* and *T. rubrum* for different antifungals were within range of 0.125µg/ml-128 µg/ml for fluconazole; 0.0313 µg/ml-32 µg/ml for itraconazole, terbinafine and griseofulvin; 0.0625 µg/ml-16 µg/ml for voriconazole and 0.000125 µg/ml-1 µg/ml for luliconazole.

To further investigate cellular responses to perturbation of ergosterol biosynthesis, we detected the expression of ERG 11 genes by real-time RT-PCR analysis. In our study, ERG 11 was
found to be up-regulated 18.18-fold in the 30 isolates. The non-responder group had a poor outcome with persistence of lesion and increased inflammation despite treatment at 4-6 weeks of clinical presentation. Up-regulation of sterol metabolism gene, ERG 11, which has been demonstrated in previous reports with C. albicans, probably emphasizes the global up-regulation of sterol metabolism genes in response to ergosterol biosynthesis inhibitors.\textsuperscript{21-23} The argument for overexpression is that accumulation of an early substrate or toxic sterol by-products induces the expression of sterol metabolism genes. The rise in ERG 11 expression in this group also indicates a probability of a poor outcome with azoles. Ergosterol is an important constituent of cellular membranes and plays a fundamental role in many biological processes.\textsuperscript{24} In the biosynthesis of ergosterol with the exception of ERG 7, most of the responsive genes function downstream of ERG 11, suggesting that their induction is in response to ergosterol depletion. This regulatory manner of gene expression in the ergosterol pathway is similar to that previously reported for ketoconazole exposure of C. albicans.\textsuperscript{25} Because overexpression of the enzyme encoded by ERG 11 is known to contribute to azole resistance in some C. albicans strains,\textsuperscript{22, 24, 25} therefore the ERG gene up-regulation may have a similar effect of azole resistance in dermatophytes. In another study by Diao et al., several other genes involved in cell wall maintenance get induced in response to itraconazole treatment, including GSC2 and Mpk A involved in β-1, 3-glucan synthase.\textsuperscript{26} The potential loss of efficacy of azoles has prompted concerted efforts to discover new drugs like Amorolphine that might block fungal growth at different metabolic sites. However, which mechanism is responsible for the global up-regulation of sterol metabolism genes in response to ergosterol biosynthesis inhibitors remains unclear. Hence the differential expression of selected genes responsible for cell wall or membrane synthesis by RT-PCR can be a useful tool in associating the rising MIC with clinical failure in patients of dermatophytosis.\textsuperscript{27,28}

**Conclusion:**

*Trichophyton mentagrophytes* is more prevalent than *Trichophyton rubrum* in the population of Delhi NCR. *Trichophyton mentagrophytes* is closely associated with T. corporis and T. cruris affecting all age groups, especially in males. Azole resistance as demonstrated by high MIC and upregulated ERG11 expression in patients not responding to treatment raises serious concern regarding excessive use of azoles and warrants judicious use in clinical settings.

In conclusion, our knowledge of understanding the complexity of calcitrant dermatophytosis extensive work on the molecular biology of dermatophytes (*T. mentagrophytes* and *T. rubrum*) is still lacking, and it is difficult to indicate antifungal resistance to be entirely responsible for the current status of resistant Tinea. Our study gives a preliminary data on ERG 11 gene upregulation with a distinct difference between the responder and clinical nonresponders, though additional mutational studies will reveal the mechanism and other potential elements like efflux pump involved in antifungal resistance.
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Conflicts of Interest

The authors report no conflicts of interest.

References


