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Study of Clinico-Mycological and Molecular Profile and Antifungal Susceptibility Pattern of Dermatophytic Onycomycosis Infection in East Delhi, India

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A B S T R A C T

Cases of onycomycosis have increased over the past few decades. In the last few years, a number of newer less toxic antifungal drugs have become available for clinical use. The increased use of antifungal, often for prolonged periods, has led to the recognition of the phenomenon of acquired antifungal resistance among previously susceptible strains or species and to the increased incidence of infections with less common species. So, there is an increasing need for rapid and accurate method for antifungal susceptibility testing. In the present study antifungal susceptibility testing was done by in-vitro micro broth dilution method according to CLSI guideline (M38-A). 25 confirmed *Trichophyton rubrum* of Nails sample collected from onycomycosis patients of suspected dermatophytosis from Delhi (India). To check Minimal inhibitory concentration (MIC) was performed in micro-titer plates with U-bottom and incubated at 35° C. Reading were taken after 96 hrs of incubation for *Trichophyton rubrum*, against 6 antifungal drugs namely fluconazole, itraconazole (triazoles), terbinafine, griseofulvin, luliconazole and voriconazole. Most of the dermatophytes had uniform patterns of susceptibility to the antifungal agents tested. Low MIC values as 64µg/ml and 0.03µg/mL were found for 52%, 64%, 72%, 88%, 100% and 100% of isolates for fluconazole, itraconazole, terbinafine, griseofulvin, luliconazole and voriconazole respectively.

Introduction

Dermatophytes, a group of keratinophilic filamentous fungi responsible for causing superficial infections Dermatophytosis in humans and animals with an estimated global prevalence of approximately 20 percent as per the World Health Organization(Marques *et al.*, 2000; Weitzman *et al.*, 1995). It causes infection of the skin, hair and nails by colonization of the keratinized layers of these structures. They are more predominant in the tropical and subtropical countries; especially in the developing countries like India where the hot climate and humid weather is favorable to the acquisition and maintenance

disease caused by them. Onychomycosis forms a major burden of clinical disease caused by dermatophytes with a reported prevalence of human onychomycosis from 2% to 8% (Brillowska-Dąbrowska *et al.,* 2007) worldwide and 0.5% to 5% in India and reported upto 45 percent as detected in a five year study from North India.

Oral antifungal therapy such as triazoles (itraconazole, fluconazole, luliconazole and voriconazole}, allylamine (terbinafine}, griseofulvin are the Current systemic treatment of choice for onycomycosis that do not respond to the topical therapy (Phillip *et al.,* 2001). Antifungals remain the choice of management in reducing the fungal load, however in the recent years clinical failure has been reported up-to 25-40 percent and a 10 percent relapse in toenail onychomycosis, after cessation of therapy has lead to a set back and the need of studying are sensitive become essential.

The variable activity of these drugs leading to treatment failure has been attributed to poor patient compliance before treatment, infection with the new strain, lack of drug penetration into the nail, bioavailability, or drug interactions and resistance. In-vitro antifungal susceptibility testing provides help in optimizing the therapy and to select an effective antifungal agent for dermatophytosis. (Khan *et al.,* 2006; Santo *et al.,* 2005; Wayne PA *et al.,* 2008)

Therefore, the purpose of this study was to detect the in-vitro antifungal susceptibility testing of dermatophytes isolated from nail infections against antifungal agents like fluconazole, itraconazole, terbinafine, griseofulvin, luliconazole and voriconazole at a tertiary care hospital of East Delhi, India using the broth micro dilution method according to CLSI standards (M38-A) (Dabas *et al.,* 2017).

Materials and Methods

The present study was conducted on a total of 160 samples from clinically diagnosed onycomycosis patients, subjected to culture from 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 40% potassium hydroxide (KOH) for processing skin and 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 antibioticswith 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 (Singh *et al.,* 2003). The molecular confirmation of isolates was done by PCR and sequencing usingspecies-specific primers of *T. rubrum*(Nagao *et al.,* 2005). The isolates were subjected to antifungal sensitivity testing by the broth microdilution method as per CLSI guidelines M-38A (Koga *et al.,* 2006).

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). PCR was performed species specificprimer of *T. rubrum* forward (203bp) GACCGACGTTCCATCAGGGGT and reverse TCAGACTGACAGCTC TTCAGAG was used for amplification (Nagao K et.al 2005). 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), $MgCl_2$ 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 10slot apparatus for 30 min. Molecular marker of 100 bp was used to determine the size of the amplicons.

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). On the basis of alignment of sequences of internal transcribed spacer region ITS 1and 2 in the NCBI nucleotide database, the isolates were identified as *T. rubrum* with 99% similarity. The representative sequence obtained was submitted to gene bank database; *T. rubrum* accession number are MH497367, MH497368, MH497369.

Preparation antifungal agents as per CLSI M-38 A

Antifungal drugs

Antifungal drugs fluconazole, itraconazole, terbinafine, griseofulvin, luliconazole and voriconazole were obtained from Sigma Pvt. Ltd. Fluconazole was dissolved in sterile distilled water and all other drugs were dissolved in 100% Dimethyl Sulfoxide (Invitrogen,). Stock solutions of 1,000 µg/ml were prepared for each drug and stored at -20°C till tests were performed. All the drugs were further diluted to two fold dilutions were performed. All working solution of drugs diluted in DMSO are prepared in tubes) before transferring onto plates, where as serial dilutions of water soluble drugs are prepared directly in microtitre plate. The final concentrations ranged from 0.125 to 64 µg/mL for fluconazole, 0.03 to 16 µg/mL for, itraconazole, terbinafine, griseofulvin, 0.00012 to 0.002 µg/mL luliconazole and 0.03 to 16 µg/mL voriconazole.

Preparation of inoculums

The MIC was performed according to CLSI (M38-A) modified method in a polysterene microtiter plates with U-bottom wells. Inoculum suspensions of dermatophytes were prepared from the seven days sub-cultures grown on Sabouraud dextrose agar at 25^0C.

The fungal colonies were covered with approximately 10 mL of normal saline, and the suspensions were made by scraping the surface with the tip of a sterile loop.

The resulting mixture of conidia and hyphae fragments was withdrawn and transferred to sterile tubes and left for 15 to 20 minutes at room temperature for conidia separation then take condia for further process. The optical density of the suspensions containing conidia and hyphae fragments was read at 530 nm, adjusted to transmittance of 80% (1 to 4 X 10^6 cells/mL) and diluted with RPMI 1640 medium and MOPS (4-Morpholine propanesulfonic acid) (Sigma Chemical Co., St. Louis, Mo). Further a 1:50 dilution was prepared to obtain the final inoculum size of approximately 0.4 X 10^3 to 5 X 10^4 cells/mL. Inoculum density of the dermatophytes was verified by quantitative colony counts on Colony Counterunit.

Test procedure (Susceptibility testing)

100 µL RPMI with antifungal drug was distributed in all wells and 200 µL of RPMI was taken as growth control in the U-bottom microtiter plate. 100 µL of inoculum was added in each well except growth control well.

Growth and sterility control wells also maintained for each assay and all the tests were performed in duplicate. MICs of quality-control ATCC strain of *T. rubrum* ATCC - 28188 were used. Plates were incubated for 96 hrs at 35^0C.MICs for fluconazole, Itraconazole, terbinafine, griseofulvin, luliconazole and voriconazole strain were taken as controls (Ghannoum *et al.*, 2006).

Endpoint determination

Endpoint determination values were performed visually at 96 hrs. The lowest dilution of the drug, which inhibited the fungal growth, was taken as the MIC.

For itraconazole drugs, MICs corresponded to the lowest drug concentration that resulted in a reduction in growth of 80% inhibition in compared with their growth control.

Terbinafine MICs corresponded to the lowest drug concentration that gave a reduction in growth of 100%and for Fluconazole, growth reduction upto 100 % (Ghannoum *et al.*, 2015)

Statistical analysis

MIC data were transformed to a normal distribution compared each antifungal agent with its MIC for each isolate. Geometric Mean MIC values were determined for all the isolates tested, and the MIC values at which 50% inhibition of colony by drug. (MIC50) were determined only for groups containing T.R 25 isolates. $P < 0.05$ was considered significant.

Results and Discussion

A total of 160 samples were collected from onychomycosis patients shown in figure 1 (a, b, c), out of 160 cases only 25 cases were of *Trichophyton rubrum* (T.R). The mean age of patients was 32.5±11.32 year (with the range from 18 to 71 years). The duration of dermatophytic infection ranged from 4 months to 8 years (8.7 months ± 6.11 months). Out of 160 samples, 140 were found to be KOH positive, of which 100 samples were culture positive and among them 25 were *Trichophyton rubrum*. Out of 25 patients 8% cases are fresh those having first time fungal infection, 52% cases, those whose were on treatment those are taking medicine from 1-2 yrs continuously and 40 % cases are recurrence type mean those patients were having infection again after some time at same or different nail.

All the 25 isolates were identified as *Trichophyton rubrum* on phenotypic mycological assessment shown in figure 1(d, e) with pigmentation and without pigmentation on culture plate of SDA. *T. rubrum* was the predominant pathogen isolatedfrom nail samples. 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 1(f).

On antifungal susceptibility testing of the 25 isolates, the minimum inhibitory concentration (MIC) was done against six antifungals (Fluconazole, Itraconazole, Terbinafine, Griseofulvin, Voriconazole and Luliconazole). The MIC of Fluconazole, Itraconazole, Terbinafine, Griseofulvin, Voriconazole and Luliconazole was within range of from 0.125-128 µg/ml, 0.0313-32 µg/ml, 0.00012-0.002 µg/ml. MIC results was lying here in 52% low MIC and 48% high MIC for fluconazole, 64% low MIC and 36% high MIC for Itraconazole

and 72% low MIC 28% high MIC for Terbinafine, 88% low MIC and 12% high MIC for Griseofulvin, while Voriconazole and lulliconazole was 100% low MIC shown in figure 2 and table 1. We have Observed that Fluconazole was more high MIC i.e. 48% as compared to Itraconazole which is 36%, Followed by Terbinafine and Griseofulvin for other antifungals among the T.R for all isolates.

Although Itraconazole has showed the highest MIC values of all the antifungal agents tested, we verified that T. rubrum strains, one specie that cause a recalcitrant chronic disease, were more susceptible to this drug than *T. rubrum* isolates. The geometric mean were 10.85, 0.47, 0.22, 0.19, 0.0002 and 0.12 for fluconazole, Itraconazole, Terbinafine, Griseofulvin, Luliconazole and Voriconazole respectively for *T. rubrum*) shown in table 2.

Onycomycosis has emerged as a significant problem in India with several cases of reinfection and relapse and failure to the treatmentbeing reported across the country. The present study was conducted in patients with dermatophytosis having nail dystrophic, 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 (Tiballi *et al.,* 1995). 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.

Table.1 Result of in-vitro activity of 6 antifungal drugs against 25 clinical isolates of *T. rubrum* from onycomycosis patients using micro dilution method

Antifungal drugs	Higher MIC	Lower MIC	MIC 50
Fluconazole	12(48%)	13(52%)	4
Itraconazole	9(36%)	16(64%)	0.25
Terbinafine	7(28%)	18(72%)	0.0625
Griseofulvin	3(12%)	22(88%)	0.125
Voriconazole	0(0%)	25(100%)	0.125
Luliconazole	0(0%)	25(100%)	0.00025

Table.2 Analysis of 25 T.R *in-vitro* susceptibility testing of six antifungal drugs of onychomycosis patients

Analysis of 25 T.R	FLU	ITR	TRB	GRS	LULI	VORI
MEAN	34.52	1.88	1.67	0.42	0.0002	0.18
SD	36.37	3.512	3.58	0.85	0.0001	0.17
GM	10.85	0.47	0.22	0.19	0.0002	0.12
MODE	64	0.0625	0.0625	0.125	0.0005	0.125
MIC 50	4	0.25	0.0625	0.125	0.00025	0.125

Fig.1 a, b, c- Nail distrophic picture of different cases**, d, e-** culture of *T. rubrum* with pigmentation and without pigmentation on SDA plate**, f-** gel picture of *T. rubrum* PCR product at 203 bp with 100 bp ladder

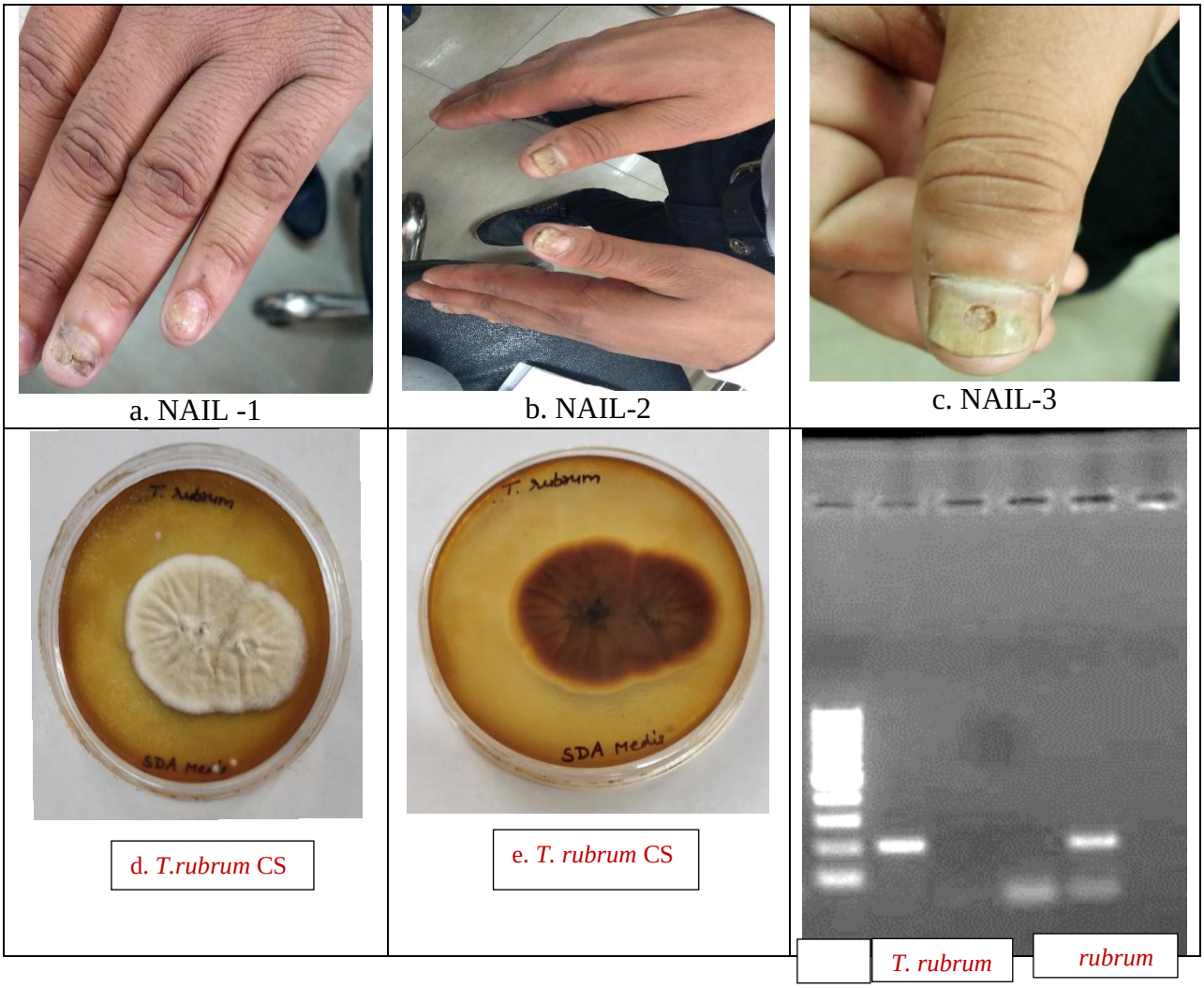
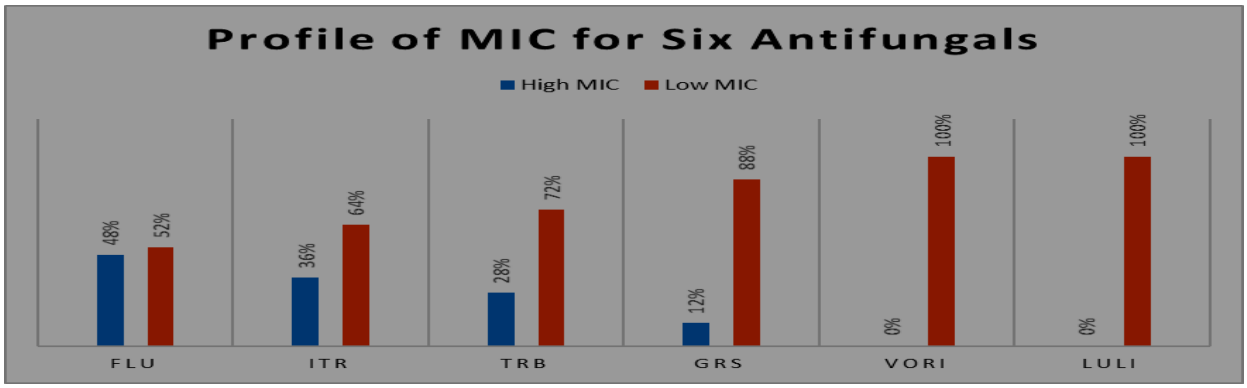


Fig.2 High MIC and low MIC against various antifungals response in onycomycosis patients for *T. rubrum*



As documented in other studies, males appeared to be more exposed to acquire dermatophytic infection (60 %) as compared to females (40 %) which is in accordance with the other researchers worldwide. Interestingly we found *T. rubrum* more prevalent species causing dermatophytosis in nails at Delhi, similar to other studies (Graser *et al.,* 1998; Diao *et al.,* 2009).

Treatment of fungal infections are less successful, than that of bacterial infections because the fungal cells being eukaryotic are similar to human than the bacteria hence drugs that inhibit or kill fungi result in toxic side effects in humans also. Moreover the fungal cells are equipped with a detoxifying system, which is able to modify many antibiotics; probably by hydroxylation. The effective antifungal drugs may extract membrane sterol, or prevent their synthesis. Most antifungal compounds target the formation or the function of ergosterol, an important component of the fungal cell membrane (Perfect *et al.,* 2017; Diao *et al.,* 2009).

In our work, the evaluation of in vitro susceptibility showed that the antifungal drugs tested, with exception of Itraconazole, other azoles displayed good activity against the dermatophytes. It is worth mentioning that Itraconazole and terbinafine had the lowest MIC values and geometric means as compared to fluconazole. Similar results have been verified by other authors that showed that these drugs had low MICs against dermatophytes. These low MICs indicate their promising roles in the treatment of onycomycosis (Da Silva Barros *et al.*, 2007).

Fluconazole and Terbinafine both are 60% sensitive and Itraconazole is less sensitive because in our hospital as a primary line of treatment mostly Fluconazole and Itraconazole is prescribe in onycomycosis patients, it is more effective in onycomycosis patients. So in our antifungal susceptibility testing Fluconazole and Itraconazole having high MIC as compared to other antifungal.

When patients are not responded to primary line of treatment for long time treatment then they prescribe Terbinafine as a secondary line of treatment so our 72% cases are sensitive but our 28 % cases are also resistant to Terbinafine according to MIC, but some patients are failure to treatment for fluconazole, Itraconazole and Terbinafine so they are prescribed antifungal in combination with low their conc. like Itraconazole and Terbinafine are given as a tertiary line of treatment they are effected by combination drugs.

Our results are similar to Tiballi *et al.,* (1995), who demonstrated higher MIC values of Itraconazole for *T. rubrum*. In our study only 8% cases are fresh, 52% cases are on treatment taking medicine from 1-2 years and 40% cases are in re-currence category having infection after 1-8years. Although Itraconazole has showed the highest MIC values of all the antifungal agents tested, we verified that *T. rubrum* strains, one specie that cause a recalcitrant chronic disease, were more susceptible to this drug than *T. rubrum* isolates.

The geometric mean was 10.85, 0.47, 0.22, 0.19, 0.0002 and 0.12 for fluconazole, Itraconazole, Terbinafine, Griseofulvin, Luliconazole and Voriconazole respectively for *T. rubrum*).

In conclusion, it may be useful to undertake periodical screening programs to detect the antifungal susceptibility of newer antifungal agents. Our data on the antifungal susceptibility of dermatophyte isolates may contribute to a choice of antifungal treatment to onycomycosis infections. Itraconazole and Terbinafine is considered as most potent drug.

But still the efficacy of Itraconazole and Terbinafine drug was totally dependent upon the variation of causative dermatophytic strains of particular nails infections.

Our knowledge of understanding the complexity of recalcitrant onycomycosis extensive work on the molecular biology of dermatophytes *(T. rubrum*) is still lacking, and it is difficult to indicate antifungal resistance to be entirely responsible for the current status of resistant onycomycosis.

We consider that our study on the antifungal susceptibility of dermatophytes can be beneficial for investigation of in vitro resistance of dermatophytic species, and for management of cases clinically unresponsive to treatment.

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The authors report no conflicts of interest.

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