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Original Research Article

Study of squalene monooxygenase mutations in response to higher MIC range among four dermatophytes & their phylogenetic relatedness to Tinea indotineae at Doon valley

Garima Sharma[1]*, **Daniel Alex Anand**[1]

[1] *Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu, India*

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ABSTRACT

Introduction: Antifungals reserved to, moderate & recurrent cases of mycosis. Allylamines considered as 1st line drugs & interfere with the ergosterol biosynthesis with SQLE gene. Strikingly elevated MIC leads to pathogen reassessment.

Aim & Objective: To find out species specific predominance, of dermatophytes, in demography of Uttarakhand. Their susceptibility range, molecular study; for mutations in squalene SQLE gene in relation to higher MIC & to corelate their phylogeny with previously reported genera.

Materials and Methods: Samples collected from public hospitals, including treatment failure & fresh cases, cultured at PDA for 25 days & identified under trinocular. Microdilution performed by EUCAST E.def 11 CLSI guidelines to calculate the MIC_{90}, further genera confirmed by multiplying ITS1, ITS4,18s & 28s rRNA specific primers, followed by sequencing. Homology confirmed at NCBI-FASTA by preparing a cladogram by CLUSTAL W & MEGA X.

Results: Compratively *Epidermophyton* & *Microsporum* recovered in huge quantity from higher altitudes. Clinical break points for *Trichophyton*, *Epidermophyton* & *Microsporum* subsequently for terbinafine (11.9-21.6μg/ml), for Itraconazole(0.22-1.25μl/ml) & for Fluconazole (0.12-0.22μl/ml) found much multiplied than previously reported MIC, at all 3 altitudes. SQLE was modified at aa F397L, A448T in mentagrophyte & L393F in rubrum rRNA.

Conclusion: It is difficult to find out the impact of increased MIC directly but helpful in associated pharmacokinetics & pharmacodynamics by calculating C_{max}/MIC, time of diffusion of drug & AUC/MIC ratios. PK/PD index in serum for increased MIC of antifungals more precisely to optimize antifungal therapy.

1. Introduction

According to WHO 25% of world population affected by dermatophytes where in India 6.09-27.6% from south & 62.5% (include HP & UK) population from Northreported.[1] Most of the developed & tropical climate emerging 18.2-23.2% reported from Brazil, *T.mentagrophyte* 21.6% from Greece, from USA most of the cases (90%)reported of onychomycosis.[2] Squalene epoxidase which diverges in all three groups of dermatophytes & responsible for allylamine resistance, become a striking evidence of modification in several pathogenic genes. Consistent use of Systemic antifungals like Triazoles, Allylamines& Polyenes in combination with steroids improved the pathogen resistance slowly & parallelly damaging the host immunity followed by promoting other microbial predators at skin.[3–7]

* *Corresponding author*.
E-mail address: garimasharma200@gmail.com (G. Sharma).

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2. Objective of Study

Aim of current study to co-relate the demographically preoccupied; mycosis species. Current study specifically treatment failure cases to calculate the Epidemiological cut offs (MIC_{90}) against frequently prescribed drugs for current pathogen, their molecular identification, Cladogram of their pathogenic genes to create phylogenetic relation with already available mycosis genera.

3. Materials and Methods

It was a cross-sectional & experimental study performed at derma OPDs of public hospitals & subsequently at SIST Biomedical lab Chennai. Protocol for study was approved by ethic clearance committee SIST Chennai.(Ref no 210/IRB-IBSEC/SIST 13 oct 2022). Subjects were informed & written consent has been taken before collection.

3.1. Exclusion & Inclusion criteria

Samples collected from group of patients with treatment failure & recurrence tinea, Pregnant, lactating females& kids (up to 14 yr.) were excluded from study to follow Helsinki guidelines for medical research.

3.2. Sample size & Statistics

Designed by following formula, for minimum sample size[8]

$\mathbf{n = z^2xp(1-p)/d^2}$	n= required sample size z= confidence level at 95% P= prevalence d= margin of error

Almost 500,300 & 500 (T-1250 according to population density) patients from Almora, Pauri & pithoragarh were observed subsequently, to target the highest incidences of preoccupied (Graph 1) & reoccurrence pathogen at public hospitals. Which was concluded by putting data in SPSS.After observing data, samples from dead shedding stratum corneum of well-defined margins of tinea; were collected by sterile blunt scalpel,also include infected nails & hair shafts. Concluded quantity of recovered samples was 150,170 & 90 at PDA (from July to Oct 2022, 2023 approx. 30-50 x1 for 3 months) was performed at each location species variation is clearly visible. (Graph 2)

3.3. Dermatomycosis culture & identification patterns

Dead marginal stratum corneum collected from patients, preliminary heat fixed with10% KOH & 30% DMSO at slides & observed under trinocular for confirmation of visible filaments. Positive samples, cultured at PDA in sterilized lab conditions. Subsequently replicas were plated in variety of media RPMI 1940, SDA, PDB for genus identification under trinocular stained with lactophenol blue.

White cottony Colonies of culture during monsoon in sterilized lab conditions were visible at 24-28^{0}C in autoclave after 7-9 days. As culture fully grown, color variation due to ample of spores formation in several mycosis genera.In fully grown colonies microconidia, macroconidia & spores visibly effective in genus identification, through tissue culture microscope (TC1000) series, at 10x & 22x power magnification. Hyphae, filaments, conidia including spores shape & size of cottony colonies was helpful during identification (Figure 1).

3.4. Clinical break points by CLSI 90 well microdilution experiment

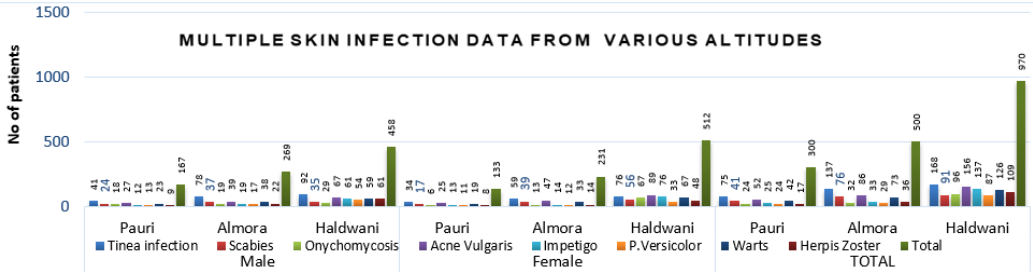
AFST performed by EUCAST method E.def 11.0,[9] pure powder of antifungals, dissolved in DMSO to prepare the stock solution (1000mg/ml). Serial 2-fold dilution which is double the final strength solution at RPMI 1640 prepared to calculate the susceptibility range of current pathogen against frequently prescribed antifungals at public hospitals. A fraction of 50μml two-fold dilutions of drug were inoculated by multichannel pipette. A disc diffusion was also performed for which single disc used at one petri plate but 2 discs were also used (for growth study only) for study of a combination of drugs as a preliminary test for pathogenic gene modifications. From middle of disc up to 14 mm was marked as zone of inhibition,15-20mm zone of sensitivity, above 22 mm zone of susceptibility was marked. Results were taken manually thrice for each species along with SD calculation (Table 1)Drug range for Itraconazole (0.125-0.20μl), Range for fluconazole (0.125-15μl), For terbinafine (0.125-35μl), Almost 0.125-35μl was experimentally designed due to previously available data from similar kind of studies.[8] 100μg of inoculum (2500-6500 CFU/ml) was added to each well & incubated for a 5-7 days at 28^{0}C, readings were taken through turbidity spectrophotometer for accuracy (Table 1). However observation of turbidity clearly visible through reading mirrors also,MIC_{90} readings were taken (Table 2). Here RPMI 1960 supplemented with cycloheximide 100mg/ml & Chloramphenicol 50mg/ml in ethanol at ph. 7 used as buffering agent . Pure antifungal powdered form was measured by the following formulas.[10]

$$Weight = \frac{Volume\,(ml) \times Conc\,(\mu g/ml)}{Assay\ Potency\,(\mu mg/gm)}$$

$$Volume = \frac{Weight\,(mg) \times Potency\,(\mu g/mg)}{Concentration\,(\mu mg/ml)}$$

3.5. Molecular level identification

Final confirmation of genus identification was done by the ITS regions.[11] DNA extraction was performed by kit method (Himedia-MB543) after crushing the fungal colonies with liquid nitrogen.For confirmation of presence



Graph 1: Above graphical presentation explaining the no of various communicable derma infections, carrying patients of both gender reach public hospitals explaining their epidemiology at all three altitudes (Pauri, Almora & Pithoragarh) of Uttarakhand.

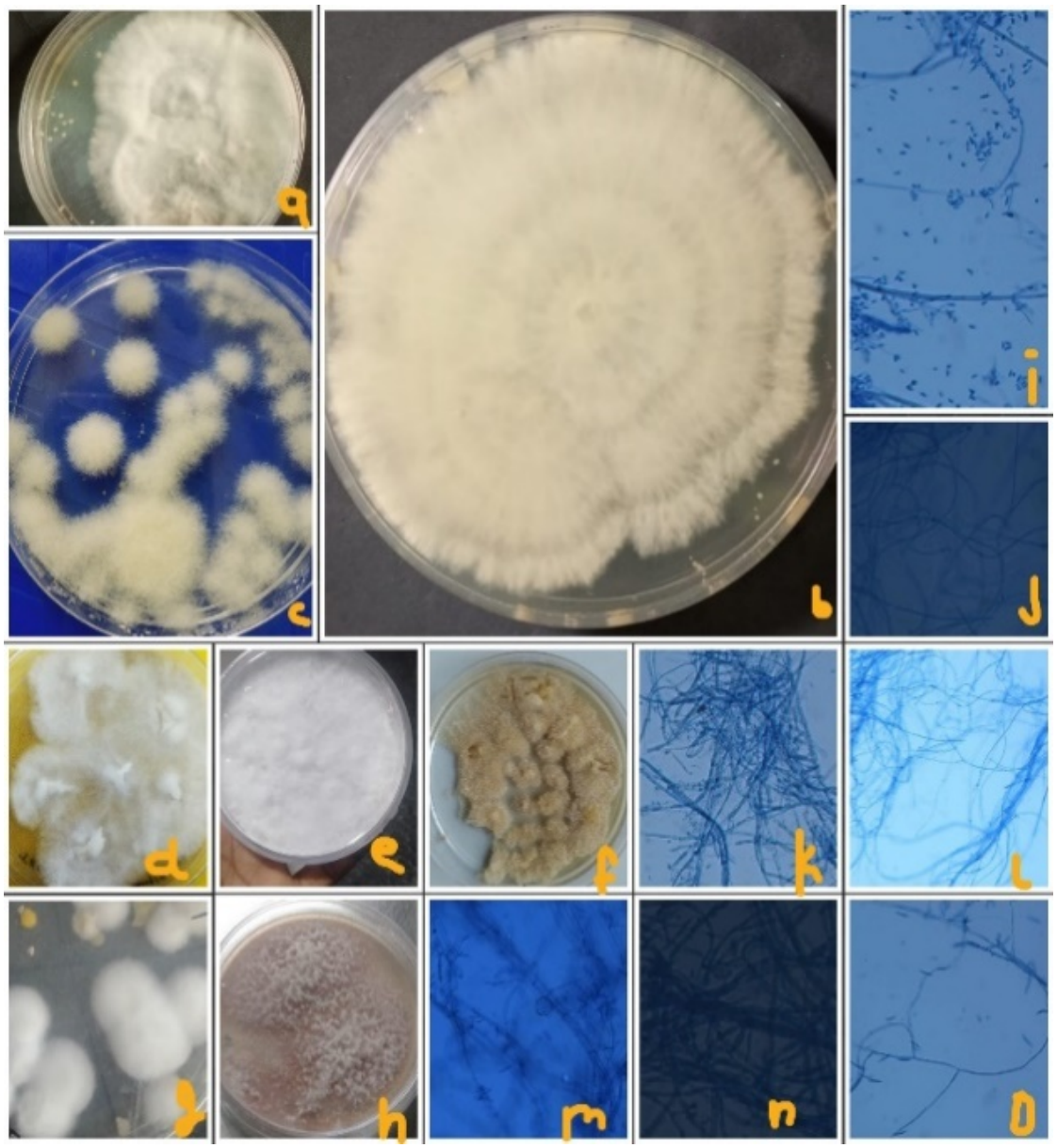
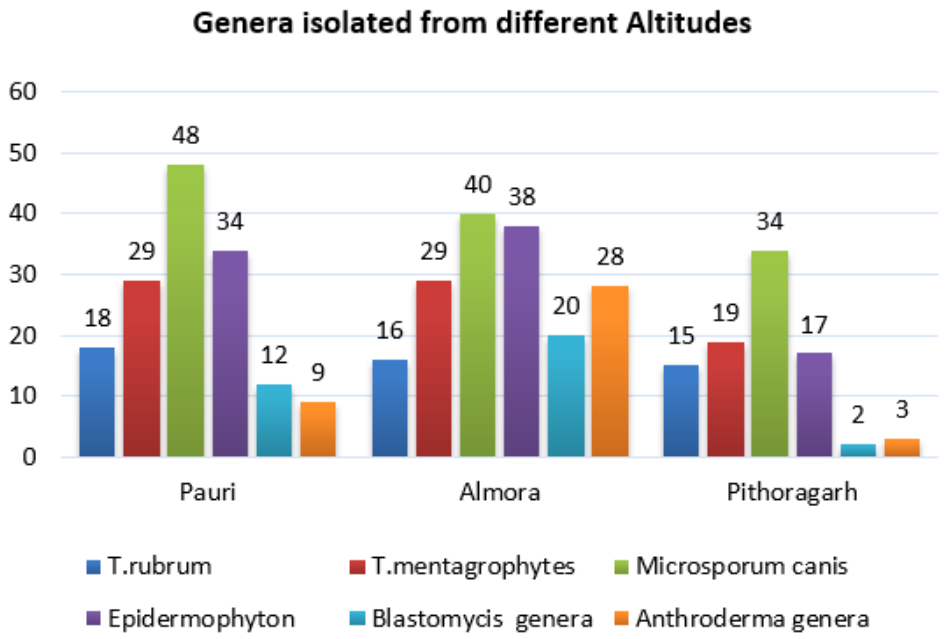


Figure 1: Mycosis collected from various heights of uttarakhand,here *T.mentagrophyte* (**a ,b** & **m**), *T.rubrum* (**d,k**-impart red pigment) collected From lower altitude in huge quantity remaining species Epidermophyto(**f,j-**turns straw colour) *microsporum* (**c,o,h,i**), Blastomycis (**e,l**) collected from higher altitudes in higher quantity.



Graph 2: Above graphica was collected from Pithoragarh, Almora & Pauri patients reach public hospitals of Almora, Haldwani & Pauri, indicating the various mycosis genera isolated from each area.

of DNA in the extraction buffer SYBR Green was used along with ITS Universal primers [12] (Forward ITS1-5′-TCCGTAGGTGAACCTGCGG-3 ReverseITS4R–5′ -TCCTCCGCTTATTGATATGC- 3′). PCR was carried out (LT-241-96 wells) in 50μl reaction volumes including 25μl of premix,3μl of DNA template,0.8μM of each primer & DDW added to maintain final volume. Reaction mixtures preheated to 98^0C for 5 min. & then 35 cycles were performed; Initially for 1 min at 96^0C, then 68^0C for 1 min & 72^0C for 1 min. followed by final extension at 72^0C for 5 minutes. The PCR products with appox.2380bp DNA were purified using a minimum elute PCR purification kit. All amplicons were evaluated at 1.5% agarose gel,loaded with ladder . After confirmation all species samples, subsequently loaded For PCR along with 28s rRNA primers & confirmed by sequencing, which primarily confirmed by the presence of successful copies in SYBR green qPCR master mix. Finally, 28s rRNA (Figure 1) was used to further confirm species. [13]

3.6. *Squalene epoxidase mutations*

To find out the alterations in SQLE gene, having exceeded MIC readings for terbinafine & other frequently prescribed antifungals were tested by the modified primers where amino acid found modified at Leu 393 Phe, Leu 393 Ser, Phe 397 leu & Gln 408 Leu.Modified primer Drsq1(5′-TTGCCAACGGGGTGTAAAG-3′) & Drsq2(5′-GGGCCATCTATAATTCAGACTC-3′) were used for rRNA multiplication of Tricophyton & *Epidermophyton*.Length of the SQLE observed was 550bp & 600bp (0ut of 1500bp) subsequently, here two strains with crossing the zone of inhibition to terbinafine was selected to PCR(as above).Both of the sequences of alignment were studied by clustal W pairwise alignment after sequencing. *Microsporum*, *E.flocossum* found 15% & *T.mentagrophytes* (KX906452), T.indotineae (OR862942) found 25% identical when compared with mutated SQLE, however with normal primer Trsq F(5'-ATGGTTGTAGAGGCTCCTCCC-3') TrsqR(5'-CTAGCTTTGAAGTTCGGCAAA3') Squalene found 82-92% similar for all the species (Figure 2b).

3.7. *Epidemiological cut off & Cladogram relatability*

It was noticed that when multiplication performed by Drsq primers & sequencing performed at Barcode Pvt. ltd. Bengaluru by sanger method. A double mutants was observed at F397Leu & Ala448Thr, after sequencing. Not only Sequence alignment but physical appearance also indicating the, *T.mentagrophytes* colonies with Indotineae. When *T.rubrum* SQLE (500bp) was sequenced one modification at Leu393Phe was also observed. After multiple sequence alignment of isolated species SQLE comparatively at FASTA NCBI; it was observed closely related & evolving toward T.Indotineae as almost 90% similar mutations were observed.(Figure 2).

Table 1:

Species	Altitude	Diameter of Zone of Sensitivity,Suseptibility &Inhibition Itraconazole	GM±SD	Fluconazole	GM±SD	Griseofulvin	GM±SD	Terbinafine
Trichophyton sp	Pith	16.0	16.06 ±0.05	20.6	20.53 ±2.87	14.7	14.86 ±0.05	0.5
	Alm	15.7		19.9		15.0		0.6
	Pau	16.3		20.8		14.9		0.5
Microsporum sp	Pith	16.8	15.95 ±0.05	19.6	19.56 ±1.82	15.2	15.26 ±0.05	0.8
	Alm	15.2		18.9		16.2		0.3
	Pau	15.9		19.3		15.9		0.7
E.flocossum sp	Pith	14.8	14.53 ±0.89	18.6	18.23 ±5.68	13.9	13.79 ±1.80	0.2
	Alm	14.2		18.2		13.2		0.6
	Pau	14.6		17.9		14.3		0.2

Disc diffusion test performed as to find out the breakpoint against antifungals as drugs crosses the zone of Inhibition (0-14mm), Zone of sensitivity (15-20mm) & Zone of susceptibility (22mm & above) .These were the average results of species occupying zones during tests from all three altitudes of Uttarakhand.*pit-Pithoragarh collection *Alm-Almora Collection *Pau-Pauri collection

Table 2:

Dermatophytes	Itraconazole (0.125-20μl)				Fluconazole (0.125-15μl)				Terbinafine (0.125-32μl)				Griseofulvin (0.125-20μl)			
	MIC_{50}	MIC_{90}	GM	SD	MIC_{50}	MIC_{90}	GM	SD	MIC_{50}	MIC_{90}	GM	SD	MIC_{50}	MIC_{90}	GM	SD
T.Mentagrophyte	0.023	0.57	0.518	± .045	0.005	0.15	0.136	±0.032	3.82	11.9	11.96	±0.62	0.12	0.72	0.672	±0.028
T.rubrum	0.027	0.63	0.612	±0.042	0.007	0.21	0.192	±0.036	4.67	12.8	14.09	±0.58	0.19	0.32	0.306	±0.022
E.flocossum	0.019	0.32	0.260	±0.052	0.083	0.25	0.212	±0.062	4.98	13.6	16.02	±0.68	0.028	0.53	0.515	±0.35
M.canis	0.002	0.058	0.057	±0.115	0.023	0.69	0.593	±0.048	3.98	12.7	11.77	±0.70	0.19	0.68	0.625	±0.40

Tabulated data was collected from patients of Almora, Pithoragarh & Pauri region reached public hospitals of Uttarakhand indicating the clinical break points Observed during experiment. Mean was calculated by 5 readings Taken by spectrophotometer, of each species & of each location, then Geometric mean taken as grand mean (Blue).

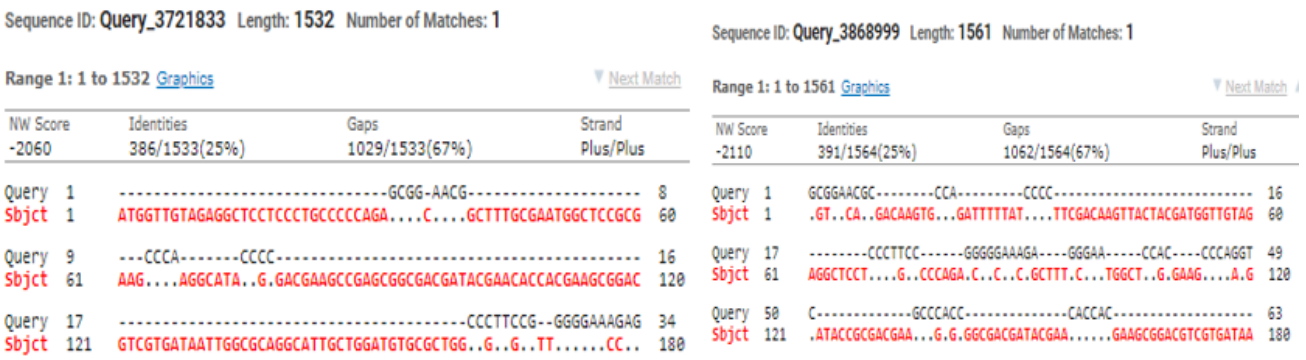


Figure 2: Squalene epoxidase of isolated species *Trichophyton* & *Microsporum* when compared to *T.indotineae* at NCBI FASTA global alignments it was found 25% similar with mutated primers.

4. Results

Compratively *Epidermophyton* & *Microsporum* recovered in huge quantity from higher altitudes. Clinical break points for *Trichophyton, Epidermophyton* & *Microsporum* subsequently for terbinafine (11.9-21.6μg/ml), for Itraconazole (0.22-1.25μl/ml) & for Fluconazole (0.12-0.22μl/ml) found much multiplied than previously reported MIC, at all 3 altitudes. SQLE was modified at aa F397L, A448T in mentagrophyte & L393F in rubrum rRNA.

5. Discussion

Current study includes six dermatophyte genera with 180 Samples, study was divided in two parts; first cross-sectional study, where Disc diffusion tests & microdilution experiments performed for confirmation of MIC_{50} &MIC_{90} range for current available pathogens including SQLE multiplicaton & sequencing of two genera. Second part contains multiplication of ITS regions & species identification. Study observes fluconazole MIC_{90}(ranges 0.15-0.69μg/ml) & Itraconazole MIC_{90}(0.058-0.78) was found slightly vary from sharma[8] study where itraconazole

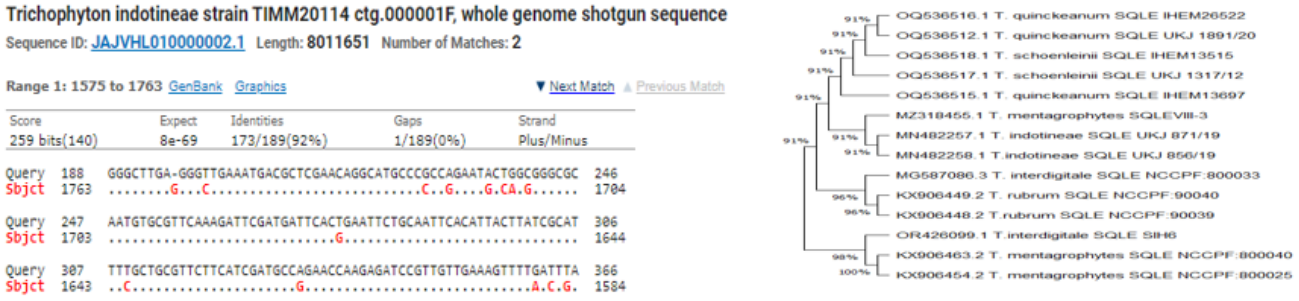


Figure 3: *Trichophyton* mentagrophytes ITS 1 & 4 were observed almost 92% similar during comparative analysis with T. indotineae, when globally align at NCBI-FASTA.(A)When cladogram was made using MEGA X with other isolated species the complete alignment toward **T.**indotineae (b).

slightly differ & fluconazole strikingly higher MIC.Here if terbinafine (12.7-11.9μg/ml)results completely contradict. However some studies like sabarter[14] supports current study where terbinafine MIC_{50} ranges between (0.06-8.0μg/ml) .Griseofulvin resistance[15–19] was also reported, & supports current study as ranges between 6.24-12.48μl/ml.[15] Current study mutations also supported by Bortoluzzi[10] found the similar kind of alterations in dermatophyte genotype. So higher ECF or MIC also indicating genotype modifications in dermatophytes. It is difficult to find out the impact of increased MIC directly but associated pharmacokinetics & pharmacodynamics by calculating C_{max}/MIC, time of diffusion of drug & AUC/MIC ratios**.**PK/PD index in serum for increased MIC of antifungals more precisely help to optimize antifungal therapy; such as help to prepare Country/Area specific dosing schedules(i.e OD,BD,TDS,SOS,HS & SOS etc in maximum permissible limit) & accordingly change in combination of drugs with higher impact & low health risk. To override current modified pathogen it is significant to find out their MIC range & PK/PD index (i.e-time of drug infusion in serum & its residing time, to maximize the impact at target organ) guiding practitioners to keep the maximum herd immunity as it is a communicable infection.

In current years from various parts of India[1] & World[11] higher MIC range & related mutations were reported, Frequently higher MIC for terbinafine leads to point mutations; F397L & L393F in T.indotineae, *T.rubrum* & *T.interdigitale*. However F415S,H440Y,I121M,V237I point mutations[20] were similar for *T.rubrum* & *T.interdigitale*. Azole efflux pumps (overexpression of TruMDR2 & Tru MDR3) resulting in azole & terbinafine higher ECF points. As up to 600bp of SQLE were studied during current study so results were similar for point mutations, from Doon valley also but current update from study observed was resistance is more toward higher altitudes.

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6. Conclusion

It is difficult to find out the impact of increased MIC directly but helpful in associated pharmacokinetics & pharmacodynamics by calculating C_{max}/MIC, time of diffusion of drug & AUC/MIC ratios. PK/PD index in serum for increased MIC of antifungals more precisely to optimize antifungal therapy.

7. Limitations

To find out the current updated status of modified SQLE of dermatophytes in this area which will be helpful for practitioners.

8. Conflicts of Interest

There was no source of funding for current study.

9. Source of Funding

None.

References

1. Gupta D, Gupta N, Deopa MS, Goyal RK. Comparison of broth microdilution method vs disc diffusion for antifungal susceptibility testing in dermatophytosis: A cross sectional study. *National J Lab Med*. 2021;10(4):18–22.
2. Verma SB, Panda S, Nenoff P, Singal A, Rudramurthy SM, Uhrlass S, et al. The unprecedented epidemic-like scenario of dermatophytosis in India: III. Antifungal resistance and treatment options. *Indian J Dermatol Venereol Leprol*. 2021;87(4):468–82.
3. Al-Hmadani AH, Al-Dhalimi MA, Alrufae MMA. Rapid Identification of Dermatophytes Isolated from Clinical Specimens from Dermatophytosis Patients by Application of the PCR-RFLP method. *Res J Pharm Biol Chem Sci*. 2016;7(4):3161–5.
4. Bhatia VK, Sharma PC. Determination of minimum inhibitory concentrations of itraconazole, terbinafine and ketoconazole against dermatophyte species by broth microdilution method. *Indian J Med Microbiol*. 2015;33(4):533–7.
5. da Fonseca L, Araújo MAS, Silva D, FM. ITS-RFLP optimization for dermatophyte identification from clinical sources in Alagoas (Brazil) versus phenotypic methods. *J Infect Dev Ctries*. 2022;16(11):1773–7.
6. Moskaluk AE, Woude SV. Current Topics in Dermatophyte Classification and Clinical Diagnosis. *Pathogens*. 2022;11(9):957. doi:10.3390/pathogens11090957.

7. Salehi Z, Shams-Gahfaroki M, Abyaneh MR. Molecular Epidemiology, Genetic Diversity, and Antifungal Susceptibility of Major Pathogenic Dermatophytes Isolated From Human Dermatophytosis. *Front Microbiol*. 2021;12:643509. doi:10.3389/fmicb.2021.64350.
8. Sacheli R, Hayette MP. Antifungal resistance in dermatophytes: Genetic considerations,clinical presentation & alternative therapies. *J Fungi (Basel)*. 2021;7(11):983. doi:10.3390/jof7110983.
9. Arendrup MC, Kahlmeter G, Guinea J, Meletiadis J. How to: perform antifungal susceptibility testing of microconidia-forming dermatophytes following the new reference EUCAST method E.Def 11.0, exemplified by Trichophyton. *Clin Microbiol Infect*. 2021;27(1):55–60.
10. Kumar P, Ramchandran S, Das S, Bhattacharya S. Insights in to changing dermatophyte spectrum in India through analysis of cumulative 165,245 cases between 1939 &2021. *Mycopathologia*. 2023;188(3):183–202.
11. Bortoluzi P, Prigitano A, Sechi A, Boneschi V, Germiniasi F, Pavan G, et al. Report of terbinafine resistant Trichophyton spp. in Italy: Clinical presentations, molecular identification, antifungal susceptibility testing and mutations in the squalene epoxidase gene. *Mycoses*. 2023;66(8):680–7.
12. Farmazi S, Motamedi M, Ali RM, Ansari S, Didehar M, Bahadoran M, et al. A simple multiplex polymerase chain reaction assay for rapid identification of the common pathogenic dermatophytes. *Curr Med Mycol*. 2021;7(2):1–7.
13. Burmester A, Uta-Christina H, Uhrla S, Nenoff P, Singal A, Verma SB. Indian Trichophyton mentagrophytes squalene epoxidase erg1 double mutants show high proportion of combined fluconazole and terbinafine resistance. *Mycoses*. 2020;63(11):1175–80.
14. Bortoluzzi P, Prigitano A, Sechi A, Boneschi V, Germiniasi F, Esposto M, et al. Report of terbinafine resistant Trichophyton spp. in Italy: Clinical presentations, molecular identification, antifungal susceptibility testing and mutations in the squalene epoxidase gene. *Mycoses*. 2023;66(8):680–7.
15. Sabater AM, Normand AC, Bidaud AL, Cremer G, Foulet F, Brun S, et al. Terbinafine Resistance in Dermatophytes: A French Multicenter Prospective Study. *J Fungi (Basel)*. 2022;8(3):220. doi:10.3390/jof8030220.
16. Sharma S, Maheshwari M, Thakur R, Sah S, Chauhan S. Antifungal susceptibility testing of five antifungal agents against clinically isolated dermatophyte species from a tertiary care hospital of northern india. *J Clin Diagn Res*. 2022;16(3):36–42.
17. Vineetha M, Sheeja S, Celine MI, Sadeep MS, Palackal S, Shanimole PE, et al. Profile of Dermatophytosis in a Tertiary Care Center. *Indian J Dermatol*. 2018;64(4):266–71.
18. Vanapalli S, Turpati NR, Gopal K, Raju PK, Devi B. A Clinico-mycological, Antifungal Drug Sensitivity and Therapeutic Study of Extensive Dermatophytosis in Coastal Andhra Pradesh. *Indian Dermatol Online J*. 2022;13(6):747–53.
19. Pavlovic MD, Marzouk S, Beciri L. Widespread dermatophytosis in a healthy adolescent: the first report of multidrug-resistant Trichophyton indotineae infection in the UAE. *Acta Dermatovenerol Alp Pannonica Adriat*. 2024;33(1):53–5.
20. Shehabeldine A, Hany EH, Hasanin M, Aymanef, Mosaed AL. Enchancing the antifungal activity of griseofulvin by incorpation a green By-polymer based nanocomposite. *Polymers*. 2021;13(4):542. doi:10.3390/polym13040542.

Author's biography

Garima Sharma, PhD Scholar https://orcid.org/0000-0002-8251-4798

Daniel Alex Anand, Associate Professor

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