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Alarming India-wide phenomenon of antifungal resistance in dermatophytes: A multicentre study

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Abstract

Background: An alarming increase in recalcitrant dermatophytosis has been witnessed in India over the past decade. Drug resistance may play a major role in this scenario.

Objectives: The aim of the present study was to determine the prevalence of in vitro resistance to terbinafine, itraconazole and voriconazole in dermatophytes, and to identify underlying mutations in the fungal squalene epoxidase (*SQLE*) gene.

Patients/Methods: We analysed skin samples from 402 patients originating from eight locations in India. Fungi were identified by microbiological and molecular methods, tested for antifungal susceptibility (terbinafine, itraconazole, voriconazole), and investigated for missense mutations in *SQLE*.

Results: *Trichophyton (T.) mentagrophytes* internal transcribed spacer (ITS) Type VIII was found in 314 (78%) samples. Eighteen (5%) samples harboured species identified up to the *T interdigitale/mentagrophytes* complex, and *T rubrum* was detected in 19 (5%) samples. 71% of isolates were resistant to terbinafine. The amino acid substitution Phe397Leu in the squalene epoxidase of resistant *T mentagrophytes* was highly prevalent (91%). Two novel substitutions in resistant *Trichophyton* strains, Ser395Pro and Ser443Pro, were discovered. The substitution Ala448Thr was found in terbinafine-sensitive and terbinafine-resistant isolates but was associated with increased MICs of itraconazole and voriconazole.

Conclusions: The high frequencies of terbinafine resistance in dermatophytes are worrisome and demand monitoring and further research. Squalene epoxidase substitutions between Leu393 and Ser443 could serve as markers of resistance in the future.

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KEYWORDS
epidemiology, infectious diseases, mechanisms of resistance, molecular typing, public health, tinea

1 | INTRODUCTION

Superficial dermatophytosis is an infection of the skin or nail that affects 20%-25% of the world's population.[1] Patients suffer from severe itching, sleep disturbance, stigmatisation and depression.[2,3] Over the past few years, healthcare professionals in India have witnessed a significant increase in the number of patients presenting with dermatophytosis, as well as in the number of difficult-to-treat and recalcitrant cases.[4] The latter has been attributed to multiple causes including an abuse of irrational fixed drug combination (FDC) creams containing potent steroids, an altered immune response of the host, and microbiological (in vitro) resistance of the causative fungi.[5,6]

In India, the most common fungi responsible for dermatophytosis belong to the *Trichophyton (T.) mentagrophytes/interdigitale* species complex. These are characterised by a particular genotype called *T mentagrophytes* internal transcribed spacer (ITS) Type VIII, which is endemic to India and is the cause of an epidemic of tinea cruris and tinea corporis resistant to the widely used drug terbinafine (TRB).[7-9] In vitro resistance to TRB has been reported in 17% and 32% of such isolates.[10,11] Several single point mutations in the fungal squalene epoxidase gene (*SQLE*), which encodes the target for TRB, have been recorded in *T rubrum* and *T mentagrophytes/interdigitale*.[11,12] These mutations lead to substitutions at one of the four amino acid positions Leu393, Phe397, Phe415 and His440 and were associated with TRB resistance. Recently, more substitutions were discovered, including in other countries such as Denmark.[13,14]

The objectives of our study were (a) to determine the species pattern and the prevalence of dermatophytes resistant to TRB, itraconazole (ITC) and voriconazole (VRC) on a large panel of patients from different sites in India, (b) to compare the results between these sites, and (c) to determine whether all cases resistant to TRB can be attributed to mutations in the fungal *SQLE* gene.

2 | MATERIALS AND METHODS

2.1 | Patients

A total of 402 patients with clinically suspected dermatophytosis from eight different locations in India participated in this study. Figure 1 shows the number and distribution of participants in each centre. From each patient, one skin scraping from the periphery of the infected lesion was collected between 2017 and 2019. This includes 199 patients whose species detection was performed in a previously published multicentric study.[7]

2.2 | Fungal identification

Each sample was transferred onto two culture media: Sabouraud's 4% dextrose agar (SDA) containing the antibiotic chloramphenicol, and SDA containing chloramphenicol as well as cycloheximide in order to eliminate moulds. Cultures were incubated at 28°C for at least 4 weeks and checked visually every 3 days. Criteria for the detection of dermatophytes were based on colour, texture and growth speed. Cultures fulfilling the listed criteria were subject to light microscopy using lactophenol cotton blue dye. In order to detect dermatophyte DNA, samples underwent a polymerase chain reaction using an enzyme-linked immunosorbent assay (PCR-ELISA). DNA was extracted using QIAamp® DNA Mini QIAcube Kit (Qiagen). The following probes for PCR were used: *T rubrum*, *T interdigitale/T mentagrophytes*, *Microsporum canis and T benhamiae*. Identification was further confirmed by Sanger sequencing of the ITS region (mainly partial 18.S rRNA, ITS1, 5.8S rRNA, ITS2 and partial 28.S rRNA) of the rDNA and the translation elongation factor (TEF)-1α gene.[15] For ITS amplification, the primers V9G (5′-TTACGTCCCTGCCCTTTGTA-3′) and LSU266 (5′-GCATTCCCAAACAACTCGACTC-3′) were used.[16] The TEF-1α gene was amplified using the primers EF-DermF (5′-CACATTAACTTGGTCGTTATCG-3′) and EF-DermR (5′-CATCCTTGGAGATACCAGC-3′).[15] We compared the sequences

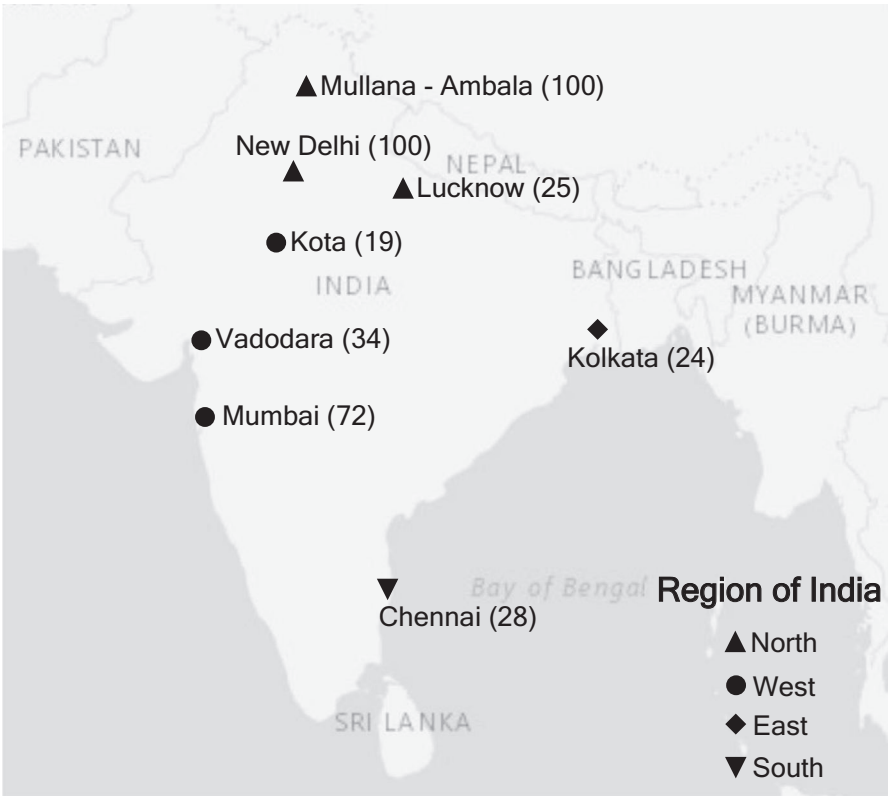


FIGURE 1 Origin of patients included in the present study. Numbers in parentheses represent the count of patients

with the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using nucleotide BLAST searches.

2.3 | Antifungal susceptibility testing

Isolated dermatophytes growing on culture media (N = 297) were tested for growth on SDA containing 0.2 μg/mL TRB as described by previous research.[12] The concentration of TRB is equivalent to twice that of the MIC for *T mentagrophytes* and *T rubrum* under these conditions.[17] Fungal growth was examined after 7 and 14 days. Growing strains were recorded as resistant. Minimal inhibitory concentrations (MICs) of TRB, ITC and VRC were determined by broth microdilution method according to the M38-A2 protocol of the Clinical and Laboratory Standards Institute (CLSI).[18]

2.4 | Squalene epoxidase gene analysis

Trichophyton total DNA was extracted from fresh fungal cultures on SDA using a DNeasy Plant minikit (Qiagen). A square-shaped area of approximately 1.0 mm^2 of growing culture was used. The squalene epoxidase (*SQLE*) gene of the TRB-resistant clinical isolates was amplified by PCR with ReadyMix Taq PCR Reaction Mix (Sigma Aldrich, Merck). The primer pair TrSQLE-F2 (5′ ATGGTTGTAGAGGCTCCTCCC 3′) and TrSQLE-R1 (5′ CTAGCTTTGAAGTTCGGCAAA 3′) was used and chromosomal DNA served as the template. In some cases where fungal cultures were not obtained, *SQLE* gene fragments were analysed from scale DNA as described[19] using primer pairs TmSQLEF4 (5′ AACGGCTTTGCGAATGGCTCC 3′) and TmSQLER4 (5′ GATGACCCTGCAGGCAGTAAG 3′). Sequences were aligned and screened for missense mutations using MEGA version 10.0.5 (Kumar, Stecher, Li, Knyaz, and Tamura 2018).

2.5 | Statistical analyses and map generation

Figures were created, Pearson's correlation coefficient was calculated, and Kruskal-Wallis tests were performed using SPSS version 26 (IBM). Fisher's exact test was executed by GraphPad Prism version 8.0.0 (GraphPad Software, www.graphpad.com). ECOFF values were computed by the Microsoft Excel spreadsheet calculator ECOFFinder XL 2010 v2.1 (https://clsi.org/meetings/microbiology/ecoffinder/), which follows a methodology established by Turnidge et al.[20] The map in Figure 1 was obtained by using the tmap package[21] within the software R and edited in Microsoft Word.

2.6 | Ethics statement and patient informed consent

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the research in this article was

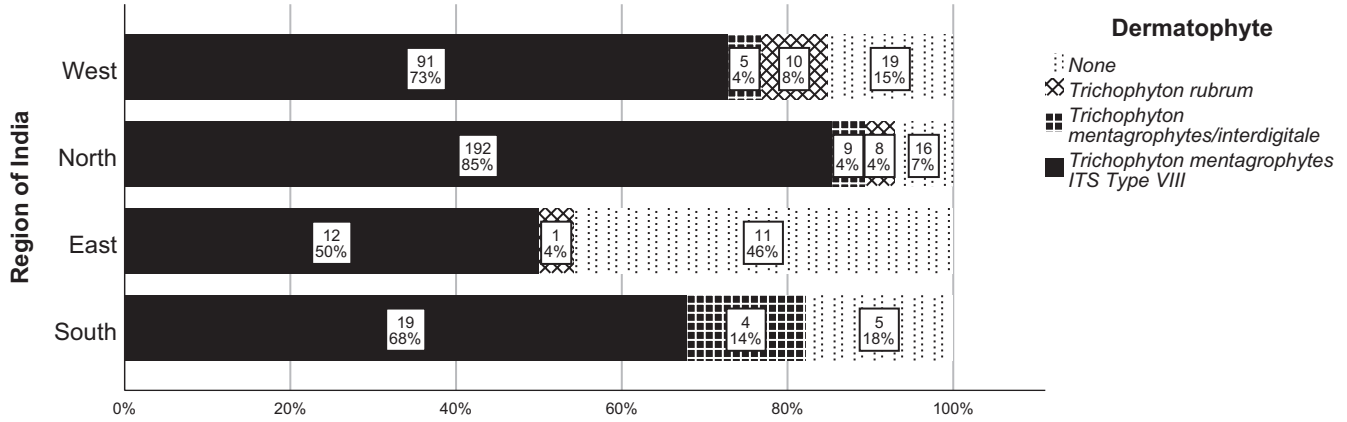


FIGURE 2 Counts and rates of detected dermatophytes in different regions of India. Detection was established by cultivation, light microscopy, PCR-ELISA, and/or sequencing of the ITS region of the rDNA or the TEF-1α gene. '*Trichophyton mentagrophytes/interdigitale*' declare strains which showed a positive PCR-ELISA result, but did not yield an ITS or TEF1-α sequence due to insufficient amounts of DNA

related to micro-organisms. All persons gave their informed consent prior to their inclusion in the study.

3 | RESULTS

3.1 | Clinical details of patients

Consistent clinical data were available for 226 (56%) patients of which 134 (59%) were male and 92 (41%) female. The ages of patients ranged from 6 to 75 years, the median age being 28 years. Tinea corporis and tinea cruris were the most common types of dermatophytoses, accounting for 196 (87%) and 185 (82%) of patients, respectively. Forty-five (20%) patients presented with tinea faciei, followed by four (2%) patients with tinea manuum, two (1%) with tinea pedis, and two (1%) with tinea barbae. The median duration of disease was 6 months and ranged from 2 weeks to 7 years. A total of 107 (47%) patients affirmed and 119 (53%) patients declined that a family member was also affected. Eleven (5%) patients indicated diabetes as comorbidity. Other pre-existing conditions such as atopy, chronic myelogenous leukaemia, chronic obstructive pulmonary disease, dyslipidaemia, hypothyroidism and vitiligo were stated by one (0.5%) patient each.

In total, 182 (81%) patients confirmed having treated their disease with pharmaceutical agents before visiting the practitioner or dermatologist. Out of these, only 83 (46%) patients could provide further information on components. Within this group, 72 (87%) had applied topical steroid creams, most often USA-class-I ultra-high-potency substances such as clobetasol propionate (50 [60%] patients) and/or class-II high-potency preparations such as betamethasone dipropionate (18 [22%] patients). Fifty-one (61%) patients who were able to specify their drug history had used topical antibiotics, most frequently gentamicin and/or ofloxacin. Exposure to topical or systemic antifungal agents was affirmed by 65 (78%) patients. Miconazole and TRB were the most widely used ingredients within this group (22 and 24 patients, respectively). Notably, 50 (60%) patients had applied topical fixed-dose combinations containing one or more ingredients in all three substance classes (steroids, antibiotics and antifungal agents).

3.2 | Microbiological investigations

All 402 samples were evaluated for growth on culture media as described in the Materials and methods section. Dermatophytes grew from 308 samples and were identified as *T mentagrophytes/interdigitale* (289 [93.8%] samples) and *T rubrum* (19 [6.2%] samples) on the base of phenotypic characters. Identification was further confirmed by PCR-ELISA and by sequencing of the ITS region and, in 147 cases, TEF1-α. All *T mentagrophytes/interdigitale* isolates revealed to be *T mentagrophytes* ITS Type VIII without exception.

Ninety-four samples that did not yield a dermatophyte growing on culture media were analysed by PCR-ELISA and sequencing directly from skin scrapings. Of these, 25 were identified as *T mentagrophytes* ITS Type VIII. In 18 samples, sequencing was negative due to insufficient amounts of DNA, but PCR-ELISA allowed an attribution to the *T interdigitale/mentagrophytes* complex. In the remaining 51 samples, no dermatophyte was detected.

Figure 2 illustrates the varying species prevalence in the different parts of India. Clearly, *T mentagrophytes* ITS Type VIII constitutes the majority of dermatophytes in all four geographic regions.

Eleven dermatophytes among the 308 we isolated were lost to contamination. Thus, 297 dermatophyte strains (279 identified as *T mentagrophytes* ITS Type VIII and 18 identified as *T rubrum*) remained for antifungal susceptibility testing and sequencing of the *SQLE* gene.

3.3 | Terbinafine resistance rates in India

A total of 297 dermatophyte isolates were tested for growth ability on SDA containing 0.2 μg/mL TRB. Terbinafine MICs were also determined by broth microdilution method (Table 1). All isolates with

TABLE 1 Terbinafine susceptibility of strains showing missense mutations in the coding region of the squalene epoxidase (*SQLE*) gene with consequential amino acid substitutions in the *SQLE* enzyme

		Broth microdilution method		Number of isolates with susceptibility determined by agar dilution method (0.2 µg/mL terbinafine)				Nucleotide accession number	Protein accession number	
		Trichophyton (T.) mentagrophytes ITS Type VIII	*T rubrum*	*T mentagrophytes* ITS Type VIII		*T rubrum*				
Missense mutation within *SQLE*	Amino acid substitution	MIC range (µg/mL)		Resistant	Sensitive	Resistant	Sensitive	*T mentagrophytes* ITS Type VIII	*T mentagrophytes* ITS Type VIII	*T rubrum*
None	None	0.125	0.0312	0	19	0	10		EZF33561	XP_003233845
Isolates with one missense mutation in SQLE										
c.1179A > C	Leu393Phe	16	-	6[9,11,22]	0	0[12,13,46]	0		AVU05317	AAQ18216
c.1178T > C	Leu393Ser	0.5-1	-	7	0	0[12,13]	0	MN519536[a]	QIJ32789[a]	-
c.1189T > C	Phe397Leu	8	8	153[9-12,22]	0	8[12,13,47]	0		AVU05318	AAZ08563
c.1191C > A										
c.1191C > G										
c.1318C > T	His440Tyr	0.25	-	2	0	0[12]	0	MN519538[a]	QIJ32791[a]	-
c.1327T > C	Ser443Pro	0.25	-	1	2	0	0	MN519539[a]	QIJ32792[a]	-
c.1342G > A	Ala448Thr	0.125	-	0	58	0	0		ATA67044	-
Isolates with two missense mutations in SQLE										
c.1005A > C	Leu335Phe	0.25	-	0	1	0	0	MN482257[a]	QIB02548[a]	-
c.1342G > A	Ala448Thr									
c.1183T > C	Ser395Pro	0.25	-	1	0	0	0	MN482258[a]	QIB02549[a]	-
c.1342G > A	Ala448Thr									
c.1189T > C	Phe397Leu	8	-	27	0	0	0		ATA67034	-
c.1191C > A										
c.1191C > G										
c.1342G > A	Ala448Thr									
c.1223A > T	Gln408Leu	1	-	2[14]	0	0	0	MN482259[a]	QIB02550[a]	-
c.1342G > A	Ala448Thr									
Total				199	80	8	10			

Note: Susceptibility was determined by broth microdilution method as well as by agar dilution method. Corresponding GenBank accession numbers of sequences are shown. Nucleotide positions apply to *SQLE* cDNA (without the intron of 62 base pairs after position 1192). Superscript numbers link to previous studies which have demonstrated an association between this substitution and terbinafine resistance of the respective species.

[a]Sequence showing a hitherto undescribed *SQLE* genotype/amino acid substitution for respective species

MIC ≥ 0.5 µg/mL (N = 203) grew well on SDA containing 0.2 µg/mL TRB, and these were considered TRB resistant. Seven strains with a MIC of 0.25 µg/mL were considered weakly resistant. Four of these strains were growing slowly on TRB agar. Dermatophytes with a MIC of 0.125 µg/mL (N = 87) or less did not grow on this medium and were considered sensitive.

Figure 3 shows the differences in frequency of TRB-resistant *T mentagrophytes* ITS Type VIII isolates depending on geographical regions. In the southern part of India, *T mentagrophytes* ITS Type VIII exhibited a much lower resistance rate (16%). In contrast, *T mentagrophytes* ITS Type VIII isolated in North, West and East India showed resistance rates in the order of 75%. The overall resistance rate of *T mentagrophytes* ITS Type VIII was much higher with 72% (202 out of 279 isolates) compared to *T rubrum* (44%, 8 out of 18 isolates).

3.4 | Amino acid substitutions in squalene epoxidase

In order to discover reasons for in vitro TRB resistance in these *Trichophyton* isolates, we screened them for alterations in the fungal squalene epoxidase gene. Missense mutations leading to substituted amino acids in the *SQLE* protein were documented and are listed in Table 1. Out of 199 *T mentagrophytes* ITS Type VIII growing on TRB agar, 180 (91%) strains had a Phe397Leu substitution and MICs of 8 µg/mL. Of these, 27 (15%) also possessed an Ala448Thr substitution. The highest MICs of 16 µg/mL were observed in isolates with Leu393Phe substitution (N = 6). Isolates with a Leu393Ser (N = 7) or Gln408Leu/Ala448Thr alteration (N = 2) showed lower MICs of 0.5-1 µg/mL. Seven isolates were weakly resistant with MICs of 0.25 µg/mL and were found with other missense mutations in the *SQLE* gene, leading to substitutions His440Tyr (N = 2), Ser443Pro (N = 3), Leu335Phe with Ala448Thr (N = 1), and Ser395Pro with Ala448Thr (N = 1). As a general rule, no MIC differences were observed for strains with the same mutation except in Leu393Ser. The commonest substitution in *SQLE* of TRB-sensitive *T mentagrophytes* ITS Type VIII was Ala448Thr (58 [72.5%] strains). This mutation did not appear to be involved in an increase in MICs.

3.5 | Triazole susceptibility

We investigated antifungal susceptibility to ITC and VRC in 297 *Trichophyton* isolates. Table 2 provides descriptive data on the drug's MICs. Significant regional differences were not observed (Table S2).

Figure 4 shows an abnormal distribution of MIC values for VRC. This observation led to the hypothesis that mechanisms of resistance exist in our cohort of isolated *T mentagrophytes* ITS Type VIII for strains with a MIC above 0.25 µg/mL. To support this value, we determined epidemiological cut-off values (ECOFF 95% and ECOFF 97.5% as described in Materials and methods) that statistically separate the wild-type population from isolates with mutational resistance. For ITR, the wild-type population was located at MIC ≤ 0.25 µg/mL, and for VRC at MIC ≤ 0.125 µg/mL.

Isolates with low sensitivity to VRC had a low sensitivity to ITC. Figure 5 shows a positive, linear relationship between in vitro susceptibilities of ITC and VRC. The calculation of a Spearman *rho* correlation coefficient yielded a strong, positive correlation between these two variables (rho (295) = 0.734, $P < .0001$).

Isolates resistant to both ITC and VRC were more prevalent in TRB-sensitive isolates (42%) than in TRB-resistant isolates (18%), assuming a MIC cut-off of 0.25 µg/mL for azoles (Table 3). Conversely, isolates resistant to TRB were more common in the azole-sensitive group (77%) than in the azole-resistant group (51%). Fisher's exact test proved these differences to be extremely statistically significant (two-tailed $P < .0001$).

Surprisingly, triazole susceptibility seemed to depend on the *SQLE* genotype. Figure 6 illustrates that the MIC means (derived from distribution curves) were higher in isolates showing an Ala448Thr substitution. We performed a Kruskal-Wallis test comparing MIC values for ITC between *Trichophyton* strains exhibiting the four most prevalent *SQLE* genotypes: wild type (N = 29),

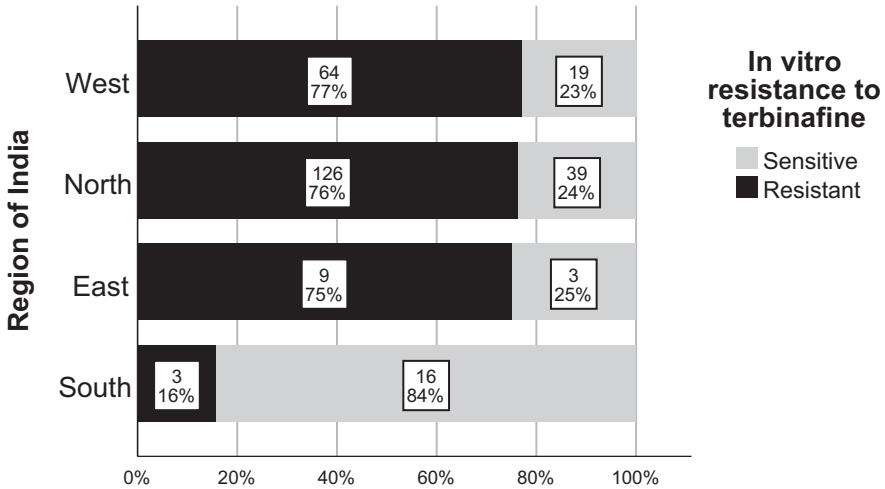


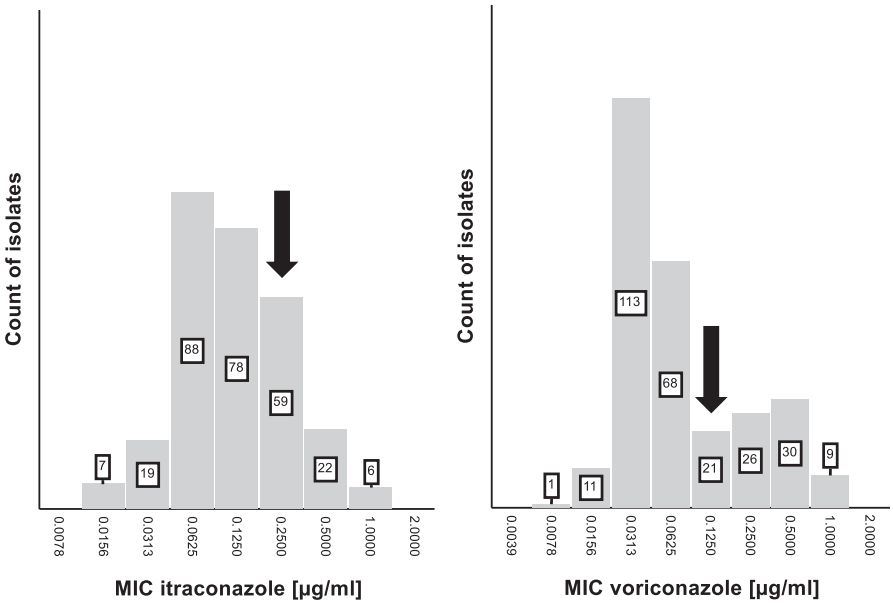
FIGURE 3 Counts and rates of terbinafine-resistant *Trichophyton mentagrophytes* ITS Type VIII in different regions of India. Resistance was determined by the ability to grow on Sabouraud's dextrose agar containing 0.2 µg/mL terbinafine and by broth microdilution method

TABLE 2 Susceptibility profile of triazoles. MIC_{50}s and MIC_{90}s denote the minimal inhibitory concentration at which at least 50% (90%) of isolates were inhibited

	Itraconazole		Voriconazole	
	MIC value for organism (µg/mL)			
Type of value	*Trichophyton (T.) mentagrophytes* ITS Type VIII	*T rubrum*	*T mentagrophytes* ITS Type VIII	*T rubrum*
N	279	18	279	18
Range	0.0156-1	0.0625-1	0.0078-1	0.0156-1
GM	0.12	0.18	0.07	0.05
MIC_{50}	0.125	0.125	0.0625	0.0312
MIC_{90}	0.25	0.5	0.5	0.5

Abbreviations: GM, geometric mean; N, number of isolates.

FIGURE 4 Susceptibility of *Trichophyton mentagrophytes* ITS Type VIII isolates to itraconazole and voriconazole, determined by broth microdilution method. Numbers in boxes indicate the count of isolates with a given minimal inhibitory concentration (MIC). Arrows denote the MIC that represents the epidemiological cut-off value (ECOFF 95% and ECOFF 97.5%)



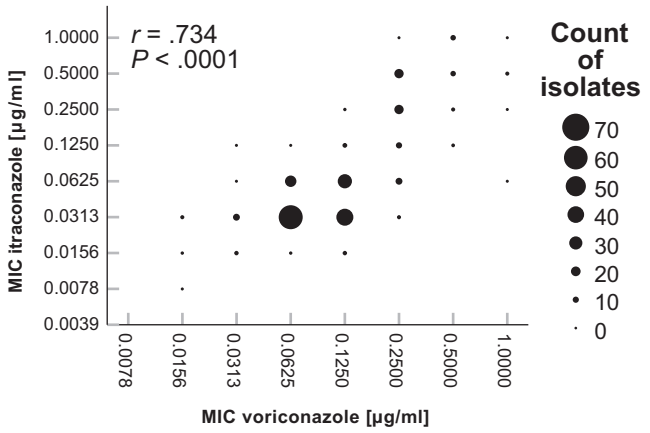


FIGURE 5 Correlation between minimal inhibitory concentrations of itraconazole and voriconazole in *Trichophyton* isolates

Phe397Leu (N = 161), Ala448Thr (N = 58) and Ala448Thr with concurrent Phe397Leu substitution (N = 27). The test yielded significant differences among genotypes ($H(3275) = 57.801$, $P < .001$). Follow-up pairwise comparisons revealed the following: Strains harbouring Ala448Thr substitution, either single or in combination with Phe397Leu, showed significantly higher MICs of ITC than strains with single Phe397Leu substitution or wild type. The same was observed for VRC: MIC values in isolates with single or combined Ala448Thr substitution were significantly higher ($H(3275) = 79.114$, $P < .001$) than in isolates exhibiting single Phe397Leu substitution or *SQLE* wild type.

TABLE 3 Count of *Trichophyton* strains resistant and sensitive to examined antifungal agents

		Terbinafine		
	In vitro resistance	Resistant	Sensitive	Total
Itraconazole and voriconazole	Resistant	33	32	65
	Sensitive	154	45	199
	Total	187	77	264

Note: Triazole resistance was more frequent in TRB-sensitive isolates, while TRB resistance was more frequent in triazole-sensitive isolates ($P < .0001$).

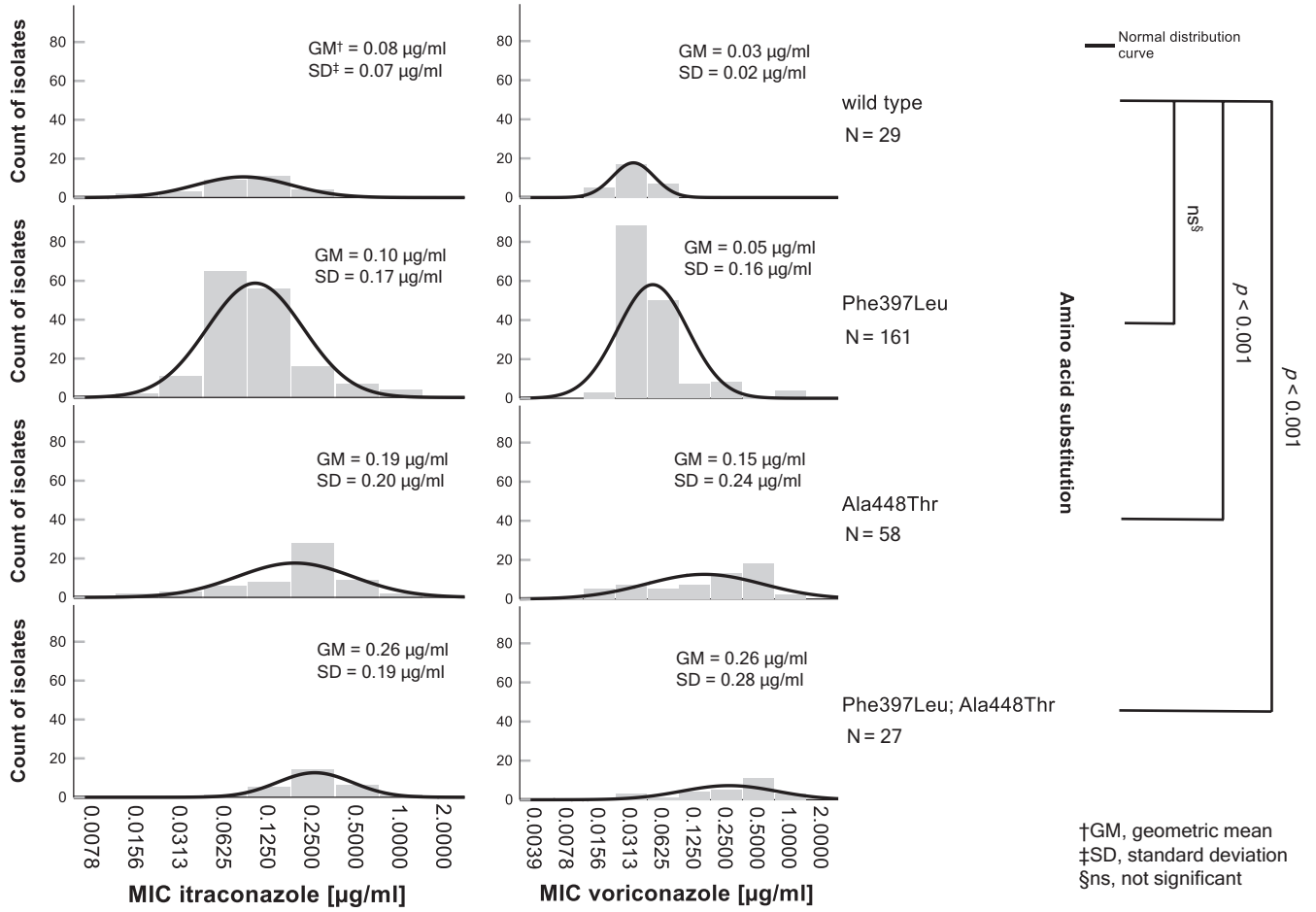


FIGURE 6 Distribution of minimal inhibitory concentrations (MICs) to itraconazole and voriconazole in the four commonest *SQLE* genotypes of *Trichophyton* isolates: wild type, Phe397Leu, Ala448Thr and Ala448Thr with concurrent Phe397Leu substitution. Isolates exhibiting an Ala448Thr substitution had significantly higher MICs

4 | DISCUSSION

4.1 | Causative pathogens for dermatophytosis

Our findings confirm *T mentagrophytes* ITS Type VIII as the commonest pathogen for dermatophytosis in all examined regions of India. This reiterates the epidemiological shift from *T rubrum* to *T mentagrophytes/T interdigitale* as the dominant pathogen in tinea patients, which has been observed in India over approximately the past decade.[7]

Furthermore, 312 (99.4%) out of 314 *T mentagrophytes* ITS Type VIII isolates showed 100% genetic concordance on the ITS region. Only two strains (0.6%) deviated from this pattern through the insertion of one base. These findings strongly suggest a clonal origin of *T mentagrophytes* strains and substantiate the epidemic-like character of the current Indian scenario of dermatophytosis.

Epidemiological studies from different locations in India have found either *T mentagrophytes* or *T interdigitale* as the dominant organism responsible for tinea.[10,11,22-27] However, a recent phylogenetic analysis of the ITS region placed Indian *T interdigitale* isolates from dermatophytosis patients in the same clade as *T mentagrophytes* ITS Type VIII presented in this study.[9] Additionally, the maximum difference between these *Trichophyton* species was only 42 single nucleotide polymorphisms (SNPs). Therefore, it can be assumed that *T mentagrophytes* ITS Type VIII and strains labelled as *T interdigitale* in recent Indian studies in fact represent the same species or genotype. However, the true anthropophilic *T interdigitale* species should be distinguished from these strains.[7]

The reason for previous divergent findings regarding the commonest species in dermatophytosis (*T mentagrophytes* vs *T interdigitale*) might lie in the process of species determination itself and differences in the used nomenclature.[28,29] It is of utmost importance for researchers to adjust submitted sequence names according to the latest taxonomic agreements, which is unfortunately not yet common practice. Failing this, confusion in species identification will continue unabated.

4.2 | Terbinafine resistance rates

The results obtained by the broth microdilution method for the identification of TRB resistance of *Trichophyton* strains were consistent with those obtained by the agar dilution method. The agar dilution method may serve as a practical alternative to the broth microdilution method as it is faster and much more cost-effective.

According to the 'Expert Consensus on The Management of Dermatophytosis in India (ECTODERM India)', TRB is recommended as the preferred systemic antifungal drug.[30] This study presents evidence that this first-line drug has lost its in vitro efficacy in most parts of India. Several studies located the prevalence of TRB-resistant isolates between 18% and 61% in different parts of India. Mahajan et al[23] reported a resistance rate of 18% (MIC ≥ 1 μg/mL) among patients infected with *T mentagrophytes* in Uttar Pradesh in 2017. A higher resistance frequency (judging from a MIC breakpoint of 2 μg/mL) of 17% in *T interdigitale* was observed in 2018 in Chandigarh, another North Indian location.[10] The same year, two studies from Delhi revealed TRB resistance rates of 32% (MIC ≥ 4 μg/mL)[11] and 61% (MIC ≥ 1 μg/mL),[22] while another research group found no resistant cases (MIC ≥ 1 μg/mL) at all.[24] Our research showed that 76% of North Indian *T mentagrophytes* isolates were resistant to TRB, exceeding the percentages of other studies in this region.

TRB resistance in *T mentagrophytes/T interdigitale* is not restricted to Northern India (Figure 3). Cities in Western India are equally affected. We also documented resistant strains in Kolkata, East India. The low resistance rate of 16% in Chennai, the capital of the southern state, Tamil Nadu, India, may be explained by the large distance (and therefore less risk of transmission of resistant strains) from northern centres showing higher percentages. It is also believed that abuse of steroid containing FDCs is less in the south of the country when compared with north.

4.3 | Associations of in vitro terbinafine resistance and amino acid substitutions in the squalene epoxidase

All isolates of *T mentagrophytes* ITS Type VIII with Phe397Leu or Leu393Phe substitution in the *SQLE* protein were shown to be resistant in agar cultures and had high MICs of ≥ 8 μg/mL. The value of 8 μg/mL was recently proposed as the epidemiological cut-off value (ECV) for TRB resistance.[31] Isolates with a non-mutated *SQLE* or carrying a single Ala448Thr substitution continuously featured low MICs of ≤0.125 μg/mL and did not grow on SDA containing 0.2 μg/mL. The *SQLE* of strains with an MIC between 0.25 and 1 μg/mL revealed other missense mutations. Of important note, a strain isolated from a patient for which TRB treatment was unsuccessful revealed a MIC of 1.0 μg/mL and a Gln408Leu substitution in *SQLE*.[14] The same value was obtained for a strain analysed in the present study. Therefore, strains with MIC ≥ 1 μg/mL can be clinically resistant to TRB.

The MICs of sensitive strains of *T mentagrophytes* isolates (0.125 μg/mL) were found to be higher than that of *T interdigitale* isolates from tinea unguium and tinea pedis in Europe.[13] These isolates had MICs of 0.008-0.03 μg/mL. The MIC of 0.125 μg/mL in the present study corresponds to the minimal value given by other authors for sensitive isolates in India.[9,11]

4.4 | Various prevalence of mutations in the SQLE gene

The high prevalence of isolates with a Phe397Leu mutation is striking while that of resistant isolates with a Leu393Phe mutation was found to be only 2%. Other missense mutations, Leu393Ser and His440Tyr, which were previously found in *T rubrum*, were revealed in *T mentagrophytes* ITS Type VIII. In addition, strains with other new mutations were discovered: Ser395Pro/Ala448Thr, Leu335Phe/Ala448Thr, Ser443Pro (all in weakly TRB-resistant strains with MIC = 0.25 μg/mL) and single Ala448Thr in TRB-sensitive strains.

Other studies have shown the existence of other substitutions associated with TRB resistance. These include Phe397Ile, Phe397Val, Phe415Ile, Phe415Ser and Phe415Val in *T rubrum*[12] and Gln408Leu in *T mentagrophytes*.[14] In addition, resistant strains were found with two mutations Ile121Met/Val237Ile and His440Tyr/Phe484Tyr in the *SQLE*.[13]

4.5 | Trends in triazole sensitivity among isolates

Triazole MICs in the present study from isolates originating in Northern India were equal to, lower,[9-11,22] and some higher[23,24] when compared with other studies.

A strong, positive, linear correlation between MICs of ITC and VRC was found. Isolates with reduced susceptibility to ITC tended to be less susceptible to VRC, and vice versa. These findings may implicate that triazole resistance in dermatophytes is not drug specific but is mediated by a shared mechanism of resistance. To the best of our knowledge, no data exist on the possible azole resistance mechanisms in *T mentagrophytes* ITS Type VIII isolates. The overexpression of two genes (multidrug resistance [MDR] 2 and MDR3) encoding a multidrug transporter of the ATP-binding cassette (ABC) family was recently documented in *T rubrum* showing reduced sensitivity to azole compounds.[32,33] Two other ABC transporters (TruMDR1 and TruMDR5) and two major facilitator superfamily (MFS) transporters (TruMFS1 and TruMFS2) were also found to be capable of operating as azole efflux pumps in *T rubrum*.[33] Orthologues of these six transporters are present in *T mentagrophytes*, and it is possible that overexpression of genes encoding multidrug transporters or missense mutations in genes encoding Cyp51A (the azole target) are involved in azole resistance.[34,35] A combination of these three mechanisms results in an additive effect. Furthermore, it has recently been discovered that mutations identified in the sterol-sensing domain of the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase-encoding gene (*hmg1*) in *Aspergillus fumigatus* resulted in dysregulation of the ergosterol biosynthesis pathway, leading to triazole resistance.[36] It is possible that similar mechanisms exist in *T mentagrophytes*.

Triazole resistance was more frequent in TRB-sensitive isolates, while TRB resistance was more frequent in triazole-sensitive isolates. This may be because patients were treated with either TRB or azole, but not with both classes of drugs. As data on pre-treatment

were only available for a fraction of patients in this study, we can neither prove nor reject this hypothesis. Investigations correlating the exposure of patients to antifungals with in vitro resistance are necessary.

We observed that reduced susceptibility to triazoles in *Trichophyton* isolates was associated with the amino acid substitution Ala448Thr at the C-terminus of the squalene epoxidase. On average, strains containing an Ala448Thr substitution exhibited higher ITC and VRC MICs. However, there are strains with the Ala448Thr substitution that showed low sensitivity to azoles. Consequently, this mutation alone cannot be incriminated for a lack of sensitivity to azoles. This observation, however, is intriguing and could have a predictive value of possible azole insensitivity. A search with InterPro,[37] a tool by the European Molecular Biology Laboratory (EMBL) for analysing conserved amino acid motifs, returned putative transmembrane helices within the squalene epoxidase. Use of the prediction program TMHMM Server 2.0[38,39] yielded the possible presence of a transmembrane helix covering the site Ala448. The change of the amino acid alanine into threonine at this position decreased prediction probability of the putative transmembrane helix using program TMHMM. It could be interpreted that such mutations influence later steps of ergosterol synthesis through conformational changes and result in reduced susceptibility to azoles. Nowadays, the identification of azole resistance mechanisms of *T mentagrophytes* ITS Type VIII remains an open field of investigation.

For dermatophytes, no clinical breakpoints (CBPs) for MIC values have yet been established to guide antifungal therapy.[40] In absence of these, epidemiological cut-off values (ECVs or ECOFFs) may serve as a temporary substitute for detection of resistance until the process of complicated CBP determination has finished.[41] During the revision of this manuscript, Shaw et al[31] published MIC data of 498 Indian *T mentagrophytes* ITS Type VIII isolates and found ECOFFs that were one dilution higher than in the present study (ECOFF (95%) ≤ 0.5 μg/mL for ITR and ECOFF (95%) ≤ 0.25 μg/mL for VRC). These ECOFFs may be helpful for physicians in the current scenario of recalcitrant dermatophytoses in India until CBPs on the basis of clinical outcome data are established.[42]

5 | CONCLUSIONS

Trichophyton mentagrophytes ITS Type VIII was confirmed as the dominant pathogen of dermatophytosis in all analysed regions of India. In vitro resistance to TRB was encountered with striking frequency in northern, western and eastern regions. These alarming findings as well as the literature cited herein underscore the urgent need for affordable and readily available facilities to check antifungal susceptibility. The absence of such investigations promotes excessively prolonged treatment, inappropriately chosen drugs, experimentation with doses and frequency, impairment of quality of life among hapless patients, and finally a significant financial loss.

The novel squalene epoxidase substitution Ala448Thr was associated with higher MICs of ITR and VRC and could have predictive value of triazole insensitivity. TRB-resistant isolates were only encountered if a substitution was located between amino acids Leu393 and Ser443 while all strains which lacked a substitution in the squalene epoxidase were sensitive to TRB in the present study. Therefore, checking specifically the C-terminus of *SQLE* allows the identification of TRB resistance in *T rubrum* or *T mentagrophytes/interdigitale*. The accuracy and feasibility of treatment decisions based on *SQLE* mutations should be evaluated in the future—especially since TRB resistance is marching across India and beyond.[43-45]

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CONFLICT OF INTEREST

There are no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Andreas Ebert: Conceptualization (equal); data curation (lead); formal analysis (equal); investigation (equal); project administration (equal); software (equal); supervision (equal); visualization (lead); writing-original draft (lead); writing-review and editing (equal). **Monod Michel**: Conceptualization (lead); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing-original draft (equal); writing-review and editing (equal). **Karine Salamin**: Investigation (equal). **Anke Burmester**: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); software (equal); writing-original draft (supporting); writing-review and editing (equal). **Silke Uhrlaß**: Data curation (equal); investigation (equal); methodology (equal); software (equal); validation (equal); **Cornelia Wiegand**: Conceptualization (equal); funding acquisition (equal); investigation (equal); project administration (equal); resources (equal); supervision (equal); writing-review and editing (equal). **Uta-Christina Hipler**: Conceptualization (equal); funding acquisition (equal); investigation (equal); project administration (equal); resources (equal); supervision (equal); writing-review and editing (equal). **Constanze Krüger**: Funding acquisition (equal); resources (equal). **Daniela Koch**: Methodology (supporting). **Franziska Wittig**: Methodology (equal). **Shyam B. Verma**: Conceptualization (equal); resources (equal); writing-review and editing (equal). **Archana Singal**: Resources (equal). **Sanjeev Gupta**: Resources (equal). **Resham Vasani**: Resources (equal). **Abir Saraswat**: Resources (equal). **Rengarajan Madhu**: Resources (equal). **Saumya Panda**: Resources (equal). **Anupam Das**: Resources (equal). **Mahendra M. Kura**: Resources (equal). **Akshy Kumar**: Resources (equal). **Shital Poojary**: Resources (equal). **Sibylle Schirm**: Formal analysis (supporting). **Yvonne Gräser**: Validation (equal). **Uwe Paasch**: Project administration (equal). **Pietro Nenoff**: Conceptualization (lead); funding acquisition (equal); investigation (equal); project administration (equal); resources (equal); supervision

(equal); validation (equal); writing-original draft (equal); writing-review and editing (equal).

DATA AVAILABILITY STATEMENT

ITS- and TEF-1α sequences of representative dermatophyte strains can be found in GenBank under accession numbers MN460830-MN460839 (*T mentagrophytes* ITS Type VIII) and MN460827-MN460829 (*T rubrum*). MN460834 and MN460835 refer to the only aberrant ITS sequences in *T mentagrophytes* ITS Type VIII found in this study, exhibiting an insertion of cytosine or guanine, respectively. *SQLE* sequences with missense mutations leading to amino acid substitutions can be obtained from Table 1. A Microsoft Excel data set containing all essential clinical and laboratory data is supplied as supporting information (Table S1).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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