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ORIGINAL ARTICLE

Reliable and rapid identification of terbinafine resistance in dermatophytic nail and skin infections

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Abstract

Background: Fungal infections are the most frequent dermatoses. The gold standard treatment for dermatophytosis is the squalene epoxidase (SQLE) inhibitor terbinafine. Pathogenic dermatophytes resistant to terbinafine are an emerging global threat. Here, we determine the proportion of resistant fungal skin infections, analyse the molecular mechanisms of terbinafine resistance, and validate a method for its reliable rapid identification.

Methods: Between 2013 and 2021, we screened 5634 consecutively isolated *Trichophyton* for antifungal resistance determined by hyphal growth on Sabouraud dextrose agar medium containing 0.2 μg/mL terbinafine. All *Trichophyton* isolates with preserved growth capacity in the presence of terbinafine underwent *SQLE* sequencing. Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method.

Results: Over an 8-year period, the proportion of fungal skin infections resistant to terbinafine increased from 0.63% in 2013 to 1.3% in 2021. Our routine phenotypic in vitro screening analysis identified 0.83% ($n = 47/5634$) of *Trichophyton* strains with in vitro terbinafine resistance. Molecular screening detected a mutation in the *SQLE* in all cases. Mutations L393F, L393S, F397L, F397I, F397V, Q408K, F415I, F415S, F415V, H440Y, or $A_{398}A_{399}G_{400}$ deletion were detected in *Trichophyton rubrum*. Mutations L393F and F397L were the most frequent. In contrast, all mutations detected in *T. mentagrophytes/T. interdigitale* complex strains were F397L, except for one strain with L393S. All 47 strains featured significantly higher MICs than terbinafine-sensitive controls. The mutation-related range of MICs varied between 0.004 and 16.0 μg/mL, with MIC as low as 0.015 μg/mL conferring clinical resistance to standard terbinafine dosing.

Conclusions: Based on our data, we propose MIC of 0.015 μg/mL as a minimum breakpoint for predicting clinically relevant terbinafine treatment failure to standard oral dosing for dermatophyte infections. We further propose growth on Sabouraud dextrose agar medium containing 0.2 μg/mL terbinafine and *SQLE* sequencing as fungal sporulation-independent methods for rapid and reliable detection of terbinafine resistance.

INTRODUCTION

Skin diseases are among the most common human illnesses, affecting at least one-third of the world population, and range depending on the country from the second to the 11th leading cause of years lived with disability according to the Global Burden of Disease Study.[1–5] Three skin conditions are among the top ten most prevalent diseases worldwide, fungal skin infections are the most common of those, and *Trichophyton rubrum*- and/or *Trichophyton interdigitale*-related dermatophytosis is a leading cause of patients seeking dermatological care.[1] Terbinafine, with both oral and topical activity, is the drug of choice for curing dermatophyte infections.[6] This antifungal, included in the List of Essential Medicines of the World Health Organization as a topical agent,[7] acts through inhibition of squalene epoxidase (SQLE), an enzyme involved in the early steps of biosynthesis of ergosterol, an essential and species-specific component of the fungal cell membrane. Terbinafine treatment leads to intracellular accumulation of squalene, which is toxic to the fungus.[8] Its excellent fungicidal therapeutic efficacy against dermatophytes, combined with its outstanding safety profile and minimal risk of drug interactions compared to azoles, renders terbinafine the gold standard for dermatophyte treatment worldwide.[9]

Inappropriate use of antibiotics, including antifungal agents, leads to the selection of treatment-resistant pathogens. Over-the-counter available antimycotics, but also patient non-compliance, treatment combinations of antifungals with topical steroids, as well as changes relative to travel and migration, are contributing to a global increase in antifungal resistance.[10,11] Documented terbinafine-resistance in *Trichophyton rubrum* was first reported in 2003.[12] The resistance in one *Trichophyton rubrum* strain was found to be caused by a missense single-point mutation in the *SQLE* gene, resulting in an amino-acid substitution, L393F.[13,14] Since then, cases of terbinafine-resistant *Trichophyton* strains have been increasing, with multiple reports from Switzerland, Germany, Finland, Denmark, Belgium, Poland, Greece and France, but also the USA, Canada, India, Iran, Vietnam, Japan, Egypt, and Brazil.[10,15–18] Strains with a terbinafine minimum inhibitory concentration (MIC) ≥ 0.125 μg/mL have been previously suggested to be considered resistant. Strains of *T. rubrum* and *T. interdigitale* have been suggested to be regarded as terbinafine-susceptible in case of MICs of >0.06 μg/mL and >0.03 μg/mL, respectively.[19] Concomitantly, numerous cases of terbinafine-resistant dermatophytosis caused by a new taxon of the *Trichophyton mentagrophytes/Trichophyton interdigitale* complex have been reported in India under either *Trichophyton interdigitale* or *Trichophyton mentagrophytes* Type VIII.[20–23] This taxon is now considered a separate species called *Trichophyton indotineae*.[23] Currently, over 70% of all dermatophyte strains of *T.rubrum* and *T. indotineae* isolated in India are terbinafine-resistant.[21]

Single-point mutations in the *SQLE* gene area are the major mechanism of terbinafine resistance. So far, substitutions at one of five amino acid positions (L393, F397, Q409, F415, and H440) within the encoded protein have been reported. Only recently was the first terbinafine-resistant strain with an amino acid deletion in the SQLE identified.[24]

The increasing antifungal resistance of dermatophytes is an emerging global health problem given the limited number of effective antifungals currently available to treat these pathogens. The objectives of this study of diagnostic and screening tests were to (i) determine the frequency of terbinafine resistance in *T. rubrum* and in species of the *T. mentagrophytes/T. interdigitale* complex in a large cohort of 5634 consecutively isolated dermatophytes from 2013 to 2021 in Switzerland, (ii) identify the underlying molecular mechanisms of drug resistance, (iii) develop and validate a diagnostic approach for a simple, rapid, and reliable identification of terbinafine-resistant strains for personalized clinical care, and (iv) determine the extent to which different molecular alterations in the fungus affect treatment outcome in patients in vivo.

MATERIALS AND METHODS

Patient data and in vivo response assessment

Skin scrapings and nails from patients with suspected dermatophyte infections were collected by physicians in the Dermatology Department of the Centre Hospitalier Universitaire Vaudois (CHUV, Lausanne University Hospital), and in dermatology practices sending samples for mycological analysis to the Dermatology Laboratory of the CHUV between 2013 and 2021. All isolated strains of *Trichophyton rubrum* and of the *T. mentagrophytes/T. interdigitale* complex were considered for this study. Patient data were collected in agreement with the VITA Certified Dermatology Biobank (CHUV-2103-12) and the Cantonal Commission on Ethics in Human Research (CER-VD 2021–00878). Strain was considered as clinically resistant to terbinafine if there was no apparent clinical response to at least one cycle of standard-dose terbinafine recommended and approved by health authorities in Switzerland (250 mg once daily for 6 weeks in case of tinea pedis, tinea manuum, tinea corporis, tinea cruris or onychomycosis of the hand and 250 mg once daily for 12 weeks in case of in case of onychomycosis of the foot).

Fungal strains and growth media

Dermatophytes were isolated as previously described.[25,26] Species identification was performed initially on the basis of the morphological appearance of the fungus in culture and microscopic observations. In all cases, where indicated, molecular fungal analysis was performed additionally. All isolated strains of *T. rubrum* and of the *T. mentagrophytes/T. interdigitale* complex were subsequently tested for fungal growth on SDA containing 0.2 μg/mL

terbinafine (SDAT).[27] A piece of SDA of approximately 0.5 × 0.5 cm with the growing dermatophytes was placed on the surface of the SDAT plate. Examination of fungal growth was performed after 7, 10, and 14 days. Glycerol stocks (15%; vol/vol) were made for all strains that had grown ($n = 47$) for further investigation.

PCR/sequencing identification of dermatophytes and *SQLE* gene analysis

The species of dermatophytes growing on SDAT was further confirmed based on a DNA sequence encoding a part of the large rRNA subunit (28S rRNA) and the internal transcribed spacer (ITS) as previously described.[28] The *SQLE* gene was sequenced after PCR amplification using the primer pair TrSQLE-F1 (5′-ATGGTTGTAGAGGCTCCTCCC-3′) and TrSQLE-R1 (5′-CTAGCTTTGAAGTTCGGCAAA-3′) and chromosomal DNA as the template.[27]

Antimicrobial susceptibility testing and minimal inhibitory concentrations

Antimicrobial susceptibility testing was performed according to guidelines for the broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2008) except for using Sabouraud dextrose broth (CM0147, Thermo Fisher Scientific) instead of RPMI1640 medium.[29] Large quantities of spores were first produced as previously described after growing the dermatophytes on 1/10 SDA plates for 14 days at 30°C as previously described.[29–31] The MIC80 was defined as the lowest concentration of terbinafine showing growth inhibition of 80% or more in comparison with absorbance values obtained without terbinafine.[30,32]

RESULTS

Phenotypic in vitro screening analysis for drug resistance to terbinafine

For this analysis, 41,513 dermatological samples referred for mycological analysis from 2013 to 2021 were screened. In 15.6% (6494/41513 clinical samples), dermatophytes were identified as pathogenic fungi. Of those, we consecutively subjected to phenotypical and molecular drug resistance analysis the complete cohort of 4229 consecutively isolated clinical strains of *T. rubrum* and 1405 strains of the *T. mentagrophytes/T. interdigitale* complex (Table 1).

To systematically screen for terbinafine resistance, we routinely assessed the growth capacity of all *T. rubrum* and strains of the *T. mentagrophytes/T. interdigitale* complex on SDA medium containing 0.2 µg/mL terbinafine (SDAT). We considered any strain showing hyphal growth to be associated with reduced in vitro sensitivity to terbinafine and potential therapeutically relevant drug resistance in vivo (Figure 1). Approximately 1% of the entire cohort of 5634 tested dermatophytes ($n = 47$; *T. rubrum* ($n = 39$) and other dermatophytes of the *T. mentagrophytes/T. interdigitale* complex ($n = 8$)) retained growth ability in the presence of terbinafine, a clue for relevant drug resistance to terbinafine in those pathogenic dermatophytes (Figure 1 and Table 1). Molecular identification revealed that the dermatophytes of the *T. mentagrophytes/T. interdigitale* complex were 5 *T.interdigitale* and 3 *T. indotineae* (Table 1). No *T. mentagrophytes* sensu stricto was found to be resistant.[33–35] Among 47 indicated patients, the mean age at diagnosis was 45.7 years (range 9–82 years). We observed a male predominance, representing 68% of the cases (32/47). Importantly, the overall proportion of *T. rubrum* and of the *T. mentagrophytes/T. interdigitale* complex strains with reduced in vitro sensitivity to terbinafine remarkably increased from 0.63% (4/630 total) in 2013 to 1.3% (8/630 total) in 2021 (Figure 2a). Consistent results were found in both *T. rubrum* (Figure 2b) and dermatophytes of the *T. mentagrophytes/T. interdigitale* complex (Figure 2c), pointing towards the growing clinical significance of antifungal-resistant dermatophytosis.

Molecular mechanisms underlying resistance to terbinafine

As terbinafine acts through inhibition of the SQLE, we sought to identify all potential genetic modifications in the *SQLE* gene that lead to fungal treatment escape. Indeed, DNA sequencing of the amplified *SQLE* revealed a point mutation that changed an amino acid at one of the five positions L393, F397, Q408, F415, and H440 within the SQLE protein in 98% of the cases ($n = 46$). *SQLE* in the one remaining strain had a nine-bp deletion leading to a three–amino acid deletion ($A_{398}A_{399}G_{400}$del). Overall, we identified the following mutations: L393F, L393S, F397L, F397I, F397V, Q408K, F415I, F415S, F415V, H440Y, and $A_{398}A_{399}G_{400}$ deletion (Figures 3 and 4). In *T. rubrum*, mutation L393F was the most frequent, representing 31% ($n = 12$) of the 39 resistant *T. rubrum* strains identified. F397L was identified in 23% ($n = 9$) of the resistant *T. rubrum*, L393S in 13% ($n = 5$), H440Y in 10% ($n = 4$), F397I in 5% ($n = 2$), and F397V in 5% ($n = 2$), while Q408K, F415I, F415S, F415V, and $A_{398}A_{399}G_{400}$ deletion were each present in 2.6% ($n = 1$) of the *T. rubrum* resistant strains (Table 1, Figure 3). The mutational landscape of the *SQLE* in the *T. mentagrophytes/T. interdigitale* complex was by far less diverse, with only two distinct point mutations identified. F397L represented 87.5% ($n = 7$) and L393S 12.5% ($n = 1$) of the eight resistant *T. mentagrophytes/T. interdigitale* complex strains. Pooled results for all growing *Trichophyton* on SDAT ranked point mutation F397L in *SQLE* as the most common resistant mutation, present in 34% of all 47 ($n = 16$) resistant strains (Table 1, Figure 3b).

TABLE 1 *T. rubrum* and *T. interdigitale* terbinafine-resistant strains in Switzerland between 2013 and 2021. Strains isolated on SDAT medium and their resistance confirmed by the MIC, determined by the broth microdilution method. Sensitive strains of *T. rubrum* and *T. interdigitale* used as controls.

Isolate No.[a]	Species	Age	Mutation	MIC terbinafine µg/mL[b]	Localization	Pretreatment with terbinafine
R1	*T. rubrum*	72	F397L	4	Onychomycosis and tinea cruris	NA
R2	*T. rubrum*	63	F397L	2	Onychomycosis	Clinically resistant to terbinafine
R3	*T. rubrum*	82	F397L	4	Tinea pedis	Clinically resistant to terbinafine
R4	*T. rubrum*	18	F397L	2	Onychomycosis	Clinically resistant to terbinafine
R5	*T. rubrum*	44	F397L	2	Onychomycosis and tinea manuum	NA
R6	*T. rubrum*	41	F397L	2	Onychomycosis	Clinically resistant to terbinafine
R7	*T. rubrum*	52	F397L	4	Onychomycosis	Clinically resistant to terbinafine
R8	*T. rubrum*	16	F397L	2	Onychomycosis	NA
R9	*T. rubrum*	32	F397L	NP	Onychomycosis	Clinically resistant to terbinafine
R10	*T. rubrum*	26	L393F	4	Tinea pedis	Clinically resistant to terbinafine
R11	*T. rubrum*	72	L393F	8	Tinea pedis	Clinically resistant to terbinafine
R12	*T. rubrum*	38	L393F	8	Tinea pedis	Clinically resistant to terbinafine
R13	*T. rubrum*	45	L393F	8	Onychomycosis	Clinically resistant to terbinafine
R14	*T. rubrum*	29	L393F	8	Onychomycosis	NA
R15	*T. rubrum*	52	L393F	8	Onychomycosis	No pretreatment
R16	*T. rubrum*	49	L393F	8	Tinea corporis	NA
R17	*T. rubrum*	38	L393F	16	Onychomycosis	NA
R18	*T. rubrum*	64	L393F	8	Onychomycosis	NA
R19	*T. rubrum*	54	L393F	8	Tinea pedis	NA
R20	*T. rubrum*	39	L393F	16	Tinea corporis	NA
R21	*T. rubrum*	72	L393F	NP	Onychomycosis	Clinically resistant to terbinafine
R22	*T. rubrum*	70	F397I	0.25	Tinea corporis	Clinically resistant to terbinafine
R23	*T. rubrum*	41	F397I	0.125	Onychomycosis	NA
R24	*T. rubrum*	44	F397V	0.062	Onychomycosis	Clinically resistant to terbinafine
R25	*T. rubrum*	56	F397V	0.004	Onychomycosis	No pretreatment
R26	*T. rubrum*	28	F415I	0.125	Onychomycosis	NA
R27	*T. rubrum*	18	F415S	NP	Tinea pedis and corporis	NA
R28	*T. rubrum*	37	F415V	0.062	Onychomycosis	NA
R29	*T. rubrum*	40	L393S	0.25	Onychomycosis	Clinically resistant to terbinafine
R30	*T. rubrum*	46	L393S	0.25	Onychomycosis	Clinically resistant to terbinafine
R31	*T. rubrum*	9	L393S	0.25	Onychomycosis	Clinically resistant to terbinafine
R32	*T. rubrum*	35	L393S	0.25	Onychomycosis	NA
R33	*T. rubrum*	14	L393S	0.25	Tinea pedis	NA
R34	*T. rubrum*	54	Q408K	0.125	Unknown	Clinically resistant to terbinafine
R35	*T. rubrum*	53	H440Y	0.0075	Onychomycosis	NA
R36	*T. rubrum*	56	H440Y	0.015	Tinea pedis	NA
R37	*T. rubrum*	49	H440Y	0.004	Onychomycosis	NA
R38	*T. rubrum*	52	H440Y	0.015	Tinea pedis et manuum	Clinically resistant to terbinafine
R39	*T. rubrum*	31	Three aa deletion ($A_{398}A_{399}G_{400}$)	0.125	Onychomycosis	Clinically resistant to terbinafine
S1	*T. rubrum*	NA	NA	0.002		
S2	*T. rubrum*	NA	NA	0.002		

TABLE 1 (Continued)

Isolate No.[a]	Species	Age	Mutation	MIC terbinafine µg/mL[b]	Localization	Pretreatment with terbinafine
S3	*T. rubrum*	NA	NA	0.002		
S4	*T. rubrum*	NA	NA	0.002		
S5	*T. rubrum*	NA	NA	0.002		
S6	*T. rubrum*	NA	NA	0.002		
R40	*T. interdigitale*	63	F397L	2	Onychomycosis	Clinically resistant to terbinafine
R41	*T. interdigitale*	33	F397L	4	Onychomycosis	NA
R42	*T. interdigitale*	65	F397L	1	Onychomycosis	NA
R43	*T. interdigitale*	47	F397L	8	Onychomycosis	No pretreatment
R44	*T. indotineae*	46	F397L	4	Tinea corporis	NA
R45	*T. indotineae*	46	F397L	2	Tinea cruris	Clinically resistant to terbinafine
R46	*T. indotineae*	51	F397L	NP	Tinea corporis	Clinically resistant to terbinafine
R47	*T. interdigitale*	65	L393S	0.125	Onychomycosis	NA
S7	*T. interdigitale*	NA	NA	0.002		
S8	*T. interdigitale*	NA	NA	0.002		
S9	*T. interdigitale*	NA	NA	0.002		
S10	*T. interdigitale*	NA	NA	0.002		

Abbreviation: NA, not available.

[a]Clinical isolates were obtained in CHUV, Lausanne, Switzerland. R: resistant; S: sensitive.

[b]NP: not possible to culture the strain (from frozen stocks) or no sporulation for retesting.

Functional relevance of mutations underlying terbinafine resistance

Broth microdilution, which allows accurate measurement of the minimal inhibitory drug concentration (MIC) necessary to prevent growth, is the gold standard method for testing microorganisms' in vitro susceptibility to antibiotics. To precisely assess the level of drug resistance of all 47 dermatophytes with detected *SQLE* mutations, we experimentally defined the MIC required to inhibit 80% of the fungal growth (MIC80) for each individual strain. The MIC values varied following the mutation (Table 1 and Table S1). Control strains that did not grow on SDAT had unmutated *SQLE* and low MICs of ≤0.002 µg/mL (mean MIC = 0.002 µg/mL, SD = 0, $n = 10$). The highest MICs were consistently measured in strains with the L393F mutation (mean MIC = 9.091 µg/mL, SD = 3.618, $n = 11$), followed by strains with the F397L mutation (mean MIC = 3.071 µg/mL, SD = 1.774, $n = 14$). The third most frequently encountered mutation, L393S, possessed a mean MIC of 0.229 µg/mL (SD = 0.051, $n = 6$) (Table S1). Strains with other missense mutations (F397I, F397V, F415I, F415S, F415V, Q408K, H440Y, three aa deletion) had lower MICs of <1 µg/mL and were regrouped together as "Other" for statistical analysis (mean MIC = 0.077 µg/mL, SD = 0.076, $n = 12$) (Table S2). Pairwise comparisons revealed statistically significant differences between control strains and all grouped strains with identified mutations in the squalene epoxidase, L393F (CI = 5.65–12.52, $p = 0.0011$), F397L (CI = 1.54–4.56, $p = 0.0011$), L393S (CI = 0.16–0.29, $p = 0.0011$), and "Other" (F397I, F397V, F415I, F415S, F415V, Q408K, H440Y, three aa deletion) (CI = 0.01–0.14, $p = 0.0011$) (Table S3).

Clinical relevance of mutations underlying terbinafine resistance

To dissect the clinical relevance of specific *SQLE* mutations and related MICs, we correlated the clinical response to terbinafine treatment with the laboratory prediction of resistance to this antifungal agent for all available data from clinical records. Clinical information was available for 22 cases (46.8%) out of 47 patients with detected *SQLE* mutations. All of these cases showed clinically relevant terbinafine resistance (Table 1). Among these, onychomycosis was the most frequent ($n = 13/22$), followed by tinea pedis ($n = 5/22$), tinea corporis ($n = 2/22$), tinea cruris ($n = 1/22$) and tinea manuum ($n = 1/22$). The MICs for strains with clinically confirmed terbinafine resistance ranged between 0.015 and 8 µg/mL. An MIC as low as 0.015 µg/mL already conferred clinical resistance to terbinafine treatment in a patient with tinea pedis and tinea manuum (case R38). Consequently, any strain growing on SDAT potentially confers clinical resistance, and the value of 0.015 µg/mL could be proposed as a minimum breakpoint for predicting terbinafine treatment failure. Switching from terbinafine to azole treatment resulted in complete resolution of the skin infection in all cases for which clinical follow-up was available (R4, R6, R10, R29, R30, R31, R33, R45, and R46). Moreover, the molecular approach to identify mutations underlying terbinafine resistance is further of high clinical relevance also for deep dermal dermatophytosis, where traditional methods may fail. Deep fungal skin infections are common in severely immunocompromised individuals, as illustrated by a recent case of a severely immunosuppressed patient, known for a grade IV malignant multimetastatic melanoma,

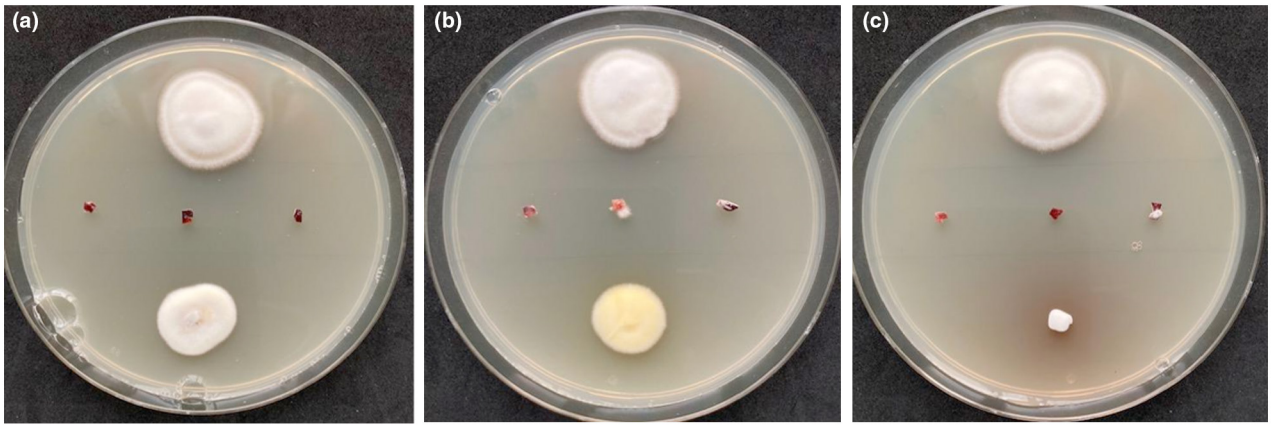

FIGURE 1 Terbinafine-resistant *Trichophyton* isolates grow on Sabouraud dextrose agar medium containing 0.2 μg/mL terbinafine. (a, b, c) Above strain: control terbinafine-resistant *T. rubrum* L393F strain (R17); middle strains: terbinafine-sensitive *T. rubrum* controls. (a) Below strain: tested terbinafine-resistant *T. rubrum* F397I strain (R23). (b) Below strain: tested terbinafine-resistant *T. rubrum* L393S strain (R31). (c) Below strain: tested terbinafine-resistant *T. rubrum* H440Y strain (R38).

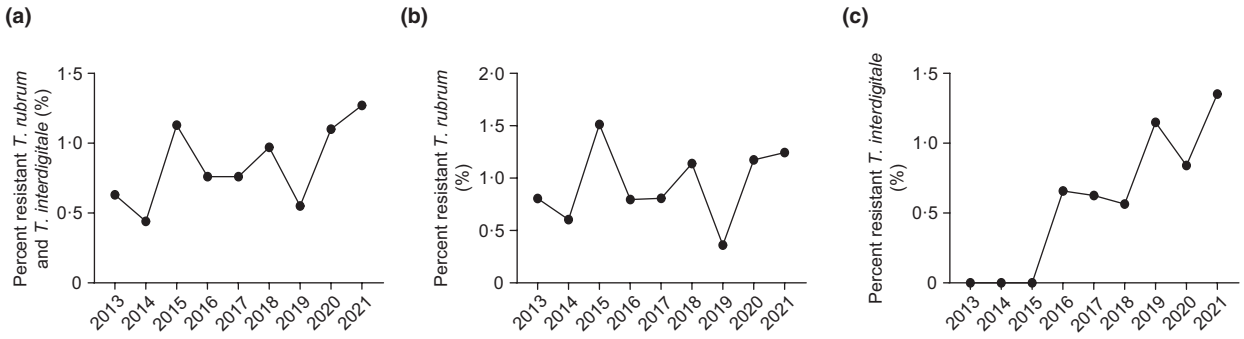

FIGURE 2 Frequency of terbinafine-resistant strains of *Trichophyton rubrum* and *T. mentagrophytes/T. interdigitale* complex in Switzerland between 2013 and 2021. (a) Terbinafine-resistant strains of *Trichophyton rubrum* and *T. mentagrophytes/T. interdigitale* complex (depicted as '*T. interdigitale*') in all *Trichophyton* analysed. (b) Terbinafine-resistant strains of *Trichophyton rubrum* in all *Trichophyton rubrum* analysed. (c) Terbinafine-resistant strains of the *T. mentagrophytes/T. interdigitale* complex (depicted as '*T.interdigitale*') in all strains of the *T. mentagrophytes/T. interdigitale* complex analysed.

who developed a deep dermatophytosis (Majocchi's granuloma; Figure 5). Histological examination combined with fungal PCR on the skin biopsy identified *Trichophyton rubrum* as the pathogenic agent. Lack of clinical response to terbinafine prompted molecular screening for terbinafine resistance, identification of F397L point mutation in the *SQLE* and switch of the systemic treatment from terbinafine to itraconazole. This resulted in immediate disease control and response to antifungal treatment after 1-month of therapy.

DISCUSSION

We have determined the prevalence of terbinafine resistance in *T. rubrum* and *T. mentagrophytes/T. interdigitale* complex in a cohort of 5634 consecutively isolated dermatophytes. We describe the molecular mechanisms underlying terbinafine resistance and validate a method that can rapidly and reliably identify terbinafine-resistant strains. Additionally, we present our own clinical experience with treatment of terbinafine-resistant infections.

Identification of terbinafine resistance in *Trichophyton rubrum* and species of the *Trichophyton mentagrophytes/T. interdigitale* complex

All except one *Trichophyton* growing on SDA medium containing 0.2 μg/mL terbinafine (SDAT) harboured a point mutation of *SQLE* between L393 and H440. Ten different point mutations were detected in *T.rubrum*. Transfer of several of these mutations into a susceptible strain of *T. rubrum* and *T. mentagrophytes* confers resistance to the fungus.[27] In addition, one strain of *T. rubrum* had a so far not reported nine-bp deletion leading to the removal of three amino acids

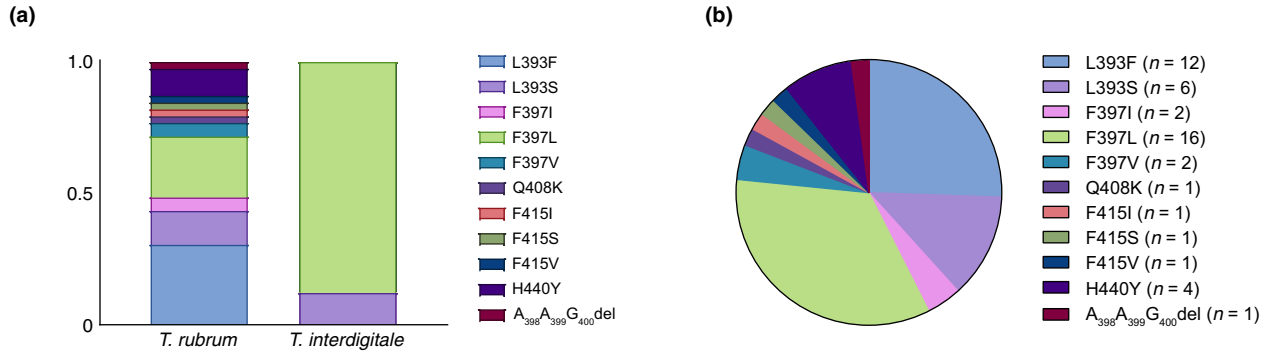

FIGURE 3 Frequency of *SQLE* mutations in terbinafine-resistant strains of *T.rubrum* and *T. mentagrophytes/T. interdigitale* complex in Switzerland between 2013 and 2021. (a) Bar plots depicting the fractions of different *SQLE* mutations in *T. rubrum* and *T. mentagrophytes/T. interdigitale* complex (depicted as '*T.interdigitale*') individually. (b) Pie chart representing overall *SQLE* mutations found in *T. rubrum* and *T. mentagrophytes/T. interdigitale* complex species combined.

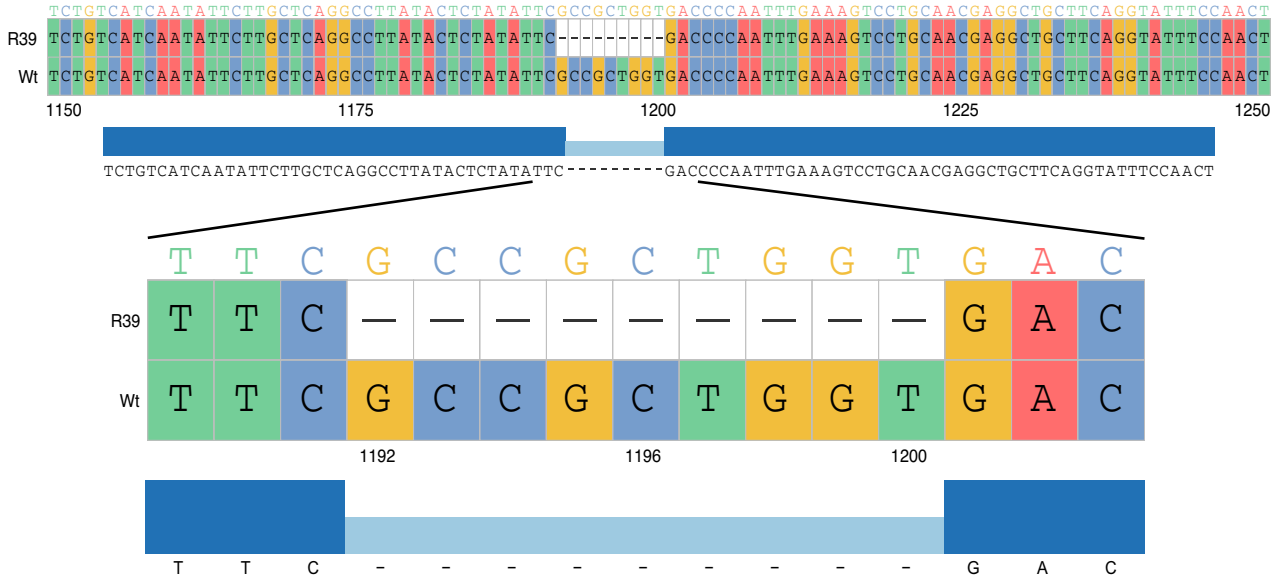

FIGURE 4 Terbinafine-resistant strain R39 shows three amino acid deletions ($A_{398}A_{399}G_{400}$del) in the SQLE. Comparison of SQLE and encoded protein sequences in wild-type *T. rubrum* and strain R39. Figure created using R package ggmsa.

($A_{398}A_{399}G_{400}$del) (Figure 4). While the F397L amino acid substitution in the SQLE was the most prevalent at 27% among resistant *T. rubrum* strains, this percentage was much lower than that for the same mutation reported in *T. indotineae* (90%).[21] All but one of the resistant strains of the *Trichophyton mentagrophytes/T. interdigitale* complex harboured F397L point mutation in the *SQLE*. Conversely, the prevalence of strains with other mutations was higher in *T. rubrum* than in *T. indotineae*, although most mutations detected in *T. rubrum* have also been recorded in *T. indotineae*.[21]

The MICs measured with the broth microdilution method varied consistently following the mutation (Table 1). The highest MICs were constantly measured in strains with the L393F mutation, followed by F397L-mutated strains (Table 1). The MICs obtained in the current study were in the range of the MICs established for *T. rubrum*[19] and for T. *indotineae*.[21] A difference of a factor of two can be explained by the fact that the MIC results were reported as MIC80, for which 80% of the strain growth was inhibited, whereas the previous results for *T. indotineae* and *T. rubrum* were reported as MIC90.[19,21] The CSLI recommendations using MIC80 for dermatophytes were adopted in the present study.

Frequency of terbinafine resistance in *Trichophyton rubrum* and species of the *Trichophyton mentagrophytes/T. interdigitale* complex

Of the large number of isolated strains in our cohort, 0.83% of *Trichophyton* (47/5634) were resistant to terbinafine over an 8-year period. A recent French multicenter prospective

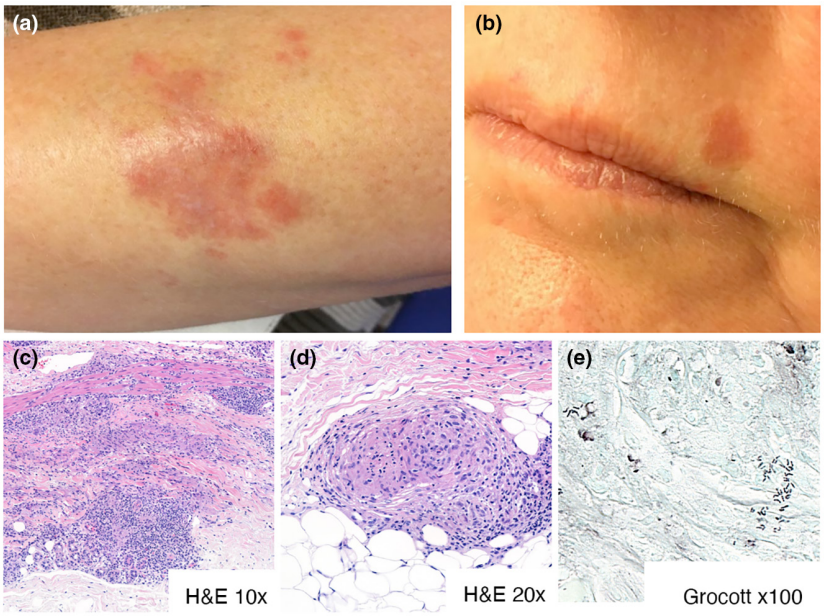

FIGURE 5 Initial clinical and histological manifestations of Majocchi's granuloma in an immunosuppressed patient. Erythematous well-delimited plaques (a) on the left forearm. (b) on the face. (c, d) Haematoxylin–Eosin staining showing important granulomatous inflammatory infiltrate with multiple multinucleated cells. 10× and 20× magnification, respectively. (e) Grocott staining showing numerous fungal filaments. 100× magnification.

study reports a resistance of 0.5%; however, these data are based on only 580 *Trichophyton* isolates over 9 months.[36] Our large-scale cohort comprising all consecutive cases of *T. rubrum* and of the *T. mentagrophytes/T. interdigitale* complex strains identified in our laboratory over an 8-year period might thus be more accurate in estimating the true resistance rates. Treatment-resistant dermatophytes are on the rise globally.[10] The rates of terbinafine-resistant dermatophytes are certainly underestimated, as susceptibility testing is not performed in routine practice. Since 2013, we have also witnessed an increase in the frequency of terbinafine-resistant strains in our study cohort, reaching 1.3% in 2021.

Simple laboratory identification of terbinafine-resistant *Trichophyton*

Antifungal sensitivity testing for *T. rubrum*, the most frequently isolated dermatophyte species in European countries, is complicated by the absent or poor sporulation of the fungus and its slow growth. In addition and in contrast to yeasts and fast-growing sporulating moulds such as *Aspergillus* spp., many strains do not grow sufficiently or at all on the synthetic RPMI 1640 medium recommended by standard techniques. Therefore, we used Sabouraud liquid medium to perform reproducible antifungal tests by the broth microdilution method.

Our data shown that SDA medium containing terbinafine allows the identification of terbinafine resistance in strains of *T. rubrum* and of the *Trichophyton mentagrophytes/T. interdigitale* complex as reliably as MICs determined by the broth microdilution method. We further show here that all strains from our study cohort growing on SDAT carry a mutation in the *SQLE* gene. Vice versa, also all strains carrying one of the mutation in the *SQLE* gene grow on SDAT. Thus, growth on SDAT as well as *SQLE* amplicon sequencing are independent of fungal sporulation and may serve as convenient and faster alternatives to the broth microdilution method for detecting terbinafine resistance and treatment decision guidance. Growth on SDAT can be easily incorporated into the routine laboratory work-up. In addition, for laboratories using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method[19] to assess antifungal susceptibility, our method could serve as a rapid screening procedure for strains requiring further evaluation with microdilution techniques.

Clinical resistance of mutated *Trichophyton rubrum* and species of the *Trichophyton mentagrophytes/T. interdigitale* complex

In all cases with detected *SQLE* mutation and available clinical records, standard-dose terbinafine treatment was shown to have no clinical effect. Among these, dermatophytosis of the nail was the most frequent diagnosis in more than half of the cases, followed by tinea pedis in five cases, and tinea corporis in two patients. As terbinafine is a lipophilic drug, it is distributed via sebum, reaching high concentrations in sebum-rich skin.[37] It might thus be difficult to compare terbinafine failure in dermatophytic infection in the sites with low sebum production, such as nails or soles, and in the glabrous skin. In the current study, the standard-dose terbinafine recommendations approved in Switzerland were used (terbinafine

250 mg once daily for 12 weeks in case of toenail dermatophytic onychomycosis and 6 weeks for dermatophytosis of the glabrous skin). Table 1 shows that an MIC value as low as 0.015 μg/mL, measured for the H440Y mutated strain from a patient with tinea pedis and manuum, already conferred clinical resistance to terbinafine. A clinical case of *T.rubrum* with a H440Y mutation in *SQLE* was previously reported.[19] Consequently, any strain growing on SDAT potentially confers clinical resistance and the value of 0.015 μg/mL can at least be proposed as a minimum breakpoint for predicting terbinafine treatment failure. Saunte and colleagues suggest strains with terbinafine MIC higher than 0.125 μg/mL to be considered as resistant.[19] Our data shows that already MICs as low as 0.015 μg/mL found in a strain with an identified H440Y mutation might be associated with both in vitro and in vivo terbinafine resistance.

Although it is difficult to compare the efficacy of a concentration in a nail plate to that of a broth medium MIC, the value of 0.015 μg/mL is higher than the concentration of terbinafine in nail clippings from healthy patients receiving 250 mg of oral terbinafine daily.[38,39] Concentrations of terbinafine ranged from 0.1 to 2.89 μg/g of nail, with mean concentrations ranging from 0.3 to 0.6 μg/g, which is 100 times higher than the MIC of a strain sensitive to terbinafine.

Consistently with previous reports[15,40] and based on our findings, a therapeutic switch to azoles cured terbinafine-resistant dermatophytosis in multiple patients. The most commonly used second-line antifungal is systemic itraconazole. In all of the terbinafine-resistant cases reporting its use in our cohort, it resulted in complete patient remission. It has been suggested that increase in terbinafine dosing (250 mg twice a day) might circumvent terbinafine resistance even in case of high MICs.[41] However, further research with larger cohorts is needed to compare efficacy and safety between standard-dose itraconazole and higher-dosed terbinafine regimen.

Dermatophytes might cause invasive infections, especially in immunocompromised individuals.[42] As numbers of patients on immunosuppressing treatment or with advanced tumours are increasing, we can reasonably expect a rise also in deep dermatophytic infections. With growing rates of resistance to standard antifungal treatment, these infections might become a true therapeutic challenge in the future.

Principles of antimicrobial stewardship have been broadly recognized by the public health community. However, unlike antibacterial resistance, antifungal stewardship has been left behind, and terbinafine-resistant dermatophytosis is now representing an emerging global health threat. Establishing precise diagnostic guidelines and optimal treatment regimens with correct dosing and duration of therapy as well as antifungal susceptibility testing should be the first step in improving the quality of antifungal use. Epidemiological surveillance and educational programmes aiming to decrease the diagnostic delay of fungal infections as well as encouraging patient compliance should be of the utmost importance in the medical community to improve patient outcomes.[43,44]

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the article or in its Appendix S1.

ETHICS STATEMENT

Discussed patient gave written informed consent for her photographs and medical information to be published in print and online and with the understanding that this information may be publicly available.

IRB STATUS

Patient data were collected in agreement with the VITA Certified Dermatology Biobank (CHUV-2103-12) and the Cantonal Commission on Ethics in Human Research (CER-VD 2021–00878).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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