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Report of terbinafine resistant *Trichophyton* spp. in Italy: Clinical presentations, molecular identification, antifungal susceptibility testing and mutations in the squalene epoxidase gene

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Abstract

Background: Numerous reports of resistance to terbinafine in *Trichophyton* spp. from all over the world are arousing justified attention and concern. Point mutations in the gene that encodes the squalene epoxidase (SQLE) enzyme are responsible for these therapeutic resistances.

Objectives: Primary objective of the study was to describe first isolates of *Trichophyton* spp. resistant to terbinafine among the patients treated between September 2019 and June 2022 at the Dermatology Units of Ospedale Maggiore Policlinico and San Bortolo Hospital. Secondary objective was to study the resistance mechanism.

Methods: Patients with confirmed *Trichophyton* spp. infection has been treated with systemic and topical terbinafine. Patients were then re-evaluated 12 weeks after the therapy. Patients with incomplete or absent response to terbinafine underwent a new skin scraping for direct mycological examination, new identification of dermatophyte species from culture and MALDI-TOF, molecular species identification, antifungal susceptibility testing and molecular analysis of SQLE gene.

Results: We identified five patients without clinical response to treatment with terbinafine. The DNA sequencing of the ITS region identified one *Trichophyton rubrum* and four *Trichophyton indotineae*. The *T. rubrum* strain showed minimum inhibitory concentration (MIC) (90% growth inhibition) of 4 mg/L for terbinafine. The four *T. indotineae* strains showed a MICs range of 0.25–4 mg/L for terbinafine. The analysis of the SQLE gene in the *T. rubrum* strain showed a nucleotide substitution generating a missense mutation (L393F). The SQLE gene sequencing in the *T. indotineae* strains showed a nucleotide substitution generating a missense mutation (F397L) in two strains, a nucleotide substitution L393S in one strain and a nucleotide substitution F415C in another strain.

A. V. Marzano and A. Grancini are senior authors.

Conclusions: We report the first cases of terbinafine-resistant *Trichophyton* isolates in the Italian population. Solid antifungal management programs will be needed to promote more responsible use of antimycotics and preserve their therapeutic efficacy to control antifungal resistance.

KEYWORDS
dermatophytes, mycology, resistance, terbinafine, Trichophyton

1 | INTRODUCTION

Dermatophytoses are the most common fungal infections in the world, with an estimated global prevalence of 20%–25%.[1] Dermatophytoses are considered mild infections, but they are highly contagious and may significantly reduce the quality of life of patients by causing itching, burning sensations, depression, stigma and sleep disturbances.[2] Infections caused by dermatophytes are usually non-lethal and easy to treat with therapy directed against the isolated species. The most common species responsible for dermatophytoses are *Trichophyton rubrum* and *Trichophyton mentagrophytes* spp. complex.[3] Terbinafine, an antifungal of the allylamine family, is the first-line drug in *Trichophyton* spp. infections and can be administered both orally and topically. This drug inhibits the enzyme squalene epoxidase (SQLE), a key enzyme in the ergosterol biosynthetic pathway. This inhibition leads to squalene accumulation, ergosterol depletion and inhibition of fungal growth.[4] In the past, it was believed that dermatophytes did not develop antifungal resistance, but unfortunately this postulate has been disproved as an epidemic of clinically widespread, recalcitrant and terbinafine-resistant dermatophytosis spread in India at the beginning of the new millennium.[5] Point mutations in the gene that encodes the SQLE enzyme are responsible for the emergence of therapeutic resistance to terbinafine. The reasons for the Indian epidemic are believed to be due to the epidemiological switch from *T. rubrum* to a new type of strain of the *T. mentagrophytes* spp. complex, initially named *T. mentagrophytes genotype VIII* and then reclassified as *Trichophyton indotineae*.[6] For some years it was thought that this was a purely Indian public health problem, but recently some reports have described cases of resistance to terbinafine have been reported in Poland, France, Belgium, Germany, Switzerland and Denmark both imported cases from endemic areas and as 'autochthonous' cases.[4,7–11] Furthermore, susceptibility testing on dermatophytes are not routinely performed in any European country and are currently reproducible only in highly specialised laboratories, suggesting that it could be a highly underestimated phenomenon. In this work we report the first five cases of clinical terbinafine-resistant strains isolated from patients attending the Dermatology Unit of the IRCCS Ca 'Granda Foundation, Ospedale Maggiore Policlinico of Milan and at the Dermatology Unit of the San Bortolo Hospital of Vicenza. In order to understand the resistance mechanism, we evaluated mutations in the gene that encodes the SQLE enzyme and performed antifungal susceptibility testing.

2 | MATERIALS AND METHODS

This study was conducted at the Dermatology Unit of the IRCCS Ca 'Granda Foundation Ospedale Maggiore Policlinico (Centre 1) and at the Dermatology Unit of the San Bortolo Hospital of Vicenza (Centre 2) from September 2019 to June 2022, in collaboration with the microbiology laboratory of the same hospitals and the Mycology Laboratory of the University of Milan.

An approval from our institutional research ethics board was obtained for data collection and written informed consent was obtained by all patients in accordance with the Italian laws regarding privacy.

All the cases involved patients aged >18 years, with a confirmed dermatophytic infection by *Trichophyton* spp., without pharmacological or anamnestic contraindications to systemic therapy with terbinafine. On the contrary, none of the patients had a diagnosis of *tinea unguium* (since the response to therapy with terbinafine cannot be clearly assessed), no therapy with systemic antifungals in the 30 days preceding the mycological sampling or localised infections not requiring systemic treatment.

For the identification of *Trichophyton* spp., skin scales specimens collected from patients with suspected dermatophytosis were inoculated on two culture media: Sabouraud dextrose agar (SDA) and SDA + chloramphenicol + cycloheximide to inhibit moulds that can mask slow growing dermatophytes. Culture plates were incubated at 28°C for at least 4 weeks and checked every 3 days. Colonies suggesting dermatophytes aspect, were evaluated for macroscopic and microscopic morphology. Culture characteristics such as surface texture and pigmentation are variable and are therefore the least reliable criteria for identification. Microscopic morphology of micro and macroconidia as well as the presence of accessory hyphal structures are the most important identification character. The slide was prepared by placing the adhesive part of a transparent tape on the colony and then attached on the slide with a drop of blue-lactophenol previously deposited. The presumptive identification was confirmed using matrix-assisted laser desorption/ionisation time of flight mass spectrometry MALDI-TOF-MS using the Vitek MS BioMerieux in the Centre 1 and the Bruker MALDI Biotyper® Sirius IVD System in the Centre 2. The correct species identification is strictly dependent on the number and quality of the spectra present in the library of the databases that could be increased by adding the spectra of standard strains in the system.

Patients have been treated with oral terbinafine at a dosage of 250 mg/day for 4 weeks and topical terbinafine for 40 days. Patients

were then re-evaluated 12 weeks after beginning of therapy. Patients with clinical incomplete or absent response to terbinafine underwent a new skin scraping for: (1) direct mycological examination, (2) new identification of dermatophyte species from culture and MALDI-TOF-MS, (3) molecular species identification using polymerase chain reaction (PCR) (amplification and sequencing of the ITS region), (4) antifungal susceptibility testing and (5) molecular analysis of SQLE gene.

2.1 | Species identification and antifungal susceptibility testing

Molecular identification was carried out for four strains. Genomic DNA was extracted using the PrepManUltra Sample Preparation Reagent (Applied Biosystems). Amplification and sequencing of the ITS region using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers was used for species-level identification.[12] Forward and reverse sequences of each isolates were analysed using Finch TV software Version 1.4.0 (www.geospizia.com). Consensus sequences of the ITS region for each sample were obtained using EMBOSS explorer (http://www.bioinformatics.nl/emboss-explorer) and aligned with the GenBank reference sequence using ClustalW pairwise alignment (https://www.ebi.ac.uk/Tools/msa/clustalo). Species identification was based on reference GeneBank sequences, KJ606115 for *T. interdigitale*, KT155896 for *T. mentagrophytes* and ON182016 for *T. indotineae*.[6]

Antifungal susceptibility testing for terbinafine and itraconazole (Sigma-Aldrich) was performed using the broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method E. Def 11.0.[13]

2.2 | Molecular analysis of squalene epoxidase gene

Genomic DNA was extracted from the cultured isolate using the PrepManUltra Sample Preparation Reagent (Applied Biosystems). A 560 bp region of the SQLE gene was amplified using Drsq1 and Drsq2 primers as reported in Salehi et al.[14] PCR was performed in a 2700 thermal cycler (Applera) set to the following conditions: denaturation at 95°C for 5 min; 30 cycles of 94°C for 30 s, 58°C for 30 s and 7°C for 45 s; and a final extension at 72°C for 5 min. PCR products were visualised on a 1.4% agarose gel stained with ethidium bromide. Amplicons were purified and sequenced with the Sanger method at Eurofins Genomics using the same primers. The forward and reverse sequences of each isolate were analysed using Finch TV software Version 1.4.0 (www.geospizia.com). Consensus sequences of the SQLE gene for each sample was obtained using EMBOSS explorer (https://www.bioinformatics.nl/emboss-explorer) and aligned with the GenBank reference sequence using ClustalW pairwise alignment (https://www.ebi.ac.uk/Tools/msa/clustalo). OM313296 and MW187977 were used as wild-type reference sequences for SQLE gene for *T. rubrum* and *T. indotineae*, respectively, as reported in Astvad et al.[15]

3 | RESULTS

We identified five patients without clinical response to treatment with terbinafine. Most of the patients were male [$n=4$ (80%)] and the mean age was 44 years (range 32–59 years). The nationality of the patients was: Italian ($n=1$), Pakistani ($n=3$), Egyptian ($n=1$). The Italian patient denied travel outside Italy in the last 10 years. All five patients had a combination of *tinea corporis* and *tinea cruris*. The clinical presentation was superimposable in all patients: large, erythematous-desquamative patches spread to the trunk and limbs, with peripheral micro-vesiculation, despite 4 weeks of systemic terbinafine therapy (Figure 1). The Italian patient was on treatment with 25 mg/day for *myasthenia gravis* secondary to a thymoma. The other four patients were in good health and were not taking any medication routinely.

Dermatophyte strains isolated in the Centre 1 were identified by MALDI-TOF Vitek MS BioMerieux as *T. rubrum* (IUM 22-0002) and *T. interdigitale* (IUM 22-0010 and IUM 22-0011), while the strains isolated in the Centre 2 were identified by MALDI-TOF-MS Bruker as *Trichophyton tonsurans* (IUM 22-0026) and *Trichophyton equinum* (IUM 22-0020). The DNA sequencing of the ITS region allowed the reclassification of the two *T. interdigitale* strains as *T. indotineae*, as well as the *T. tonsurans* and the *T. equinum* strains in *T. indotineae* (Figures 2 and 3).

The *T. rubrum* strain showed MIC values (90% growth inhibition) of 4 mg/L for terbinafine and 0.12 mg/L for itraconazole. The four *T. indotineae* strains showed a MICs (90% growth inhibition) range of 0.25–4 mg/L for terbinafine and of 0.03–0.12 mg/L for itraconazole (Table 1). There are no specific break points (BPs) for dermatophytes, but according to EUCAST's ECOFFs (Epidemiological cut-off value)[13,16] the four *T. indotineae* strains resulted above the limit of the wild type (WT-UL) for terbinafine and under the WT-UL for itraconazole, considering both 90% and 50% growth inhibition. The *T. rubrum* strain resulted under the WT-UL for itraconazole, but upper the terbinafine WT-UL considering the 90% of inhibition.

The analysis of a region of the SQLE gene in the *T. rubrum* strain showed a nucleotide substitution (1179 A>T) generating the missense mutation L393F. The SQLE gene sequencing in the *T. indotineae* strains showed a nucleotide substitution (1189 T>C) generating the missense mutation F397L in two strains, a nucleotide substitution (1178 T>C) causing the mutation L393S in one strain, and a nucleotide substitution (1244 T>G) causing the mutation F415C in another strain.

The SQLE gene sequence of the terbinafine-resistant strain 22-0002 showed 100% homology with the mutated sequence Genebank ID OM313304 of *T. rubrum*. Two isolates of *T. indotineae* (IUM 22-0010 and 22-0011) with the mutation F397L showed 100% homology with the Genebank ID OM313310 as reported in Astvad et al.,[15] while the *T. indotineae* stain (IUM 22-0020) with the L393S

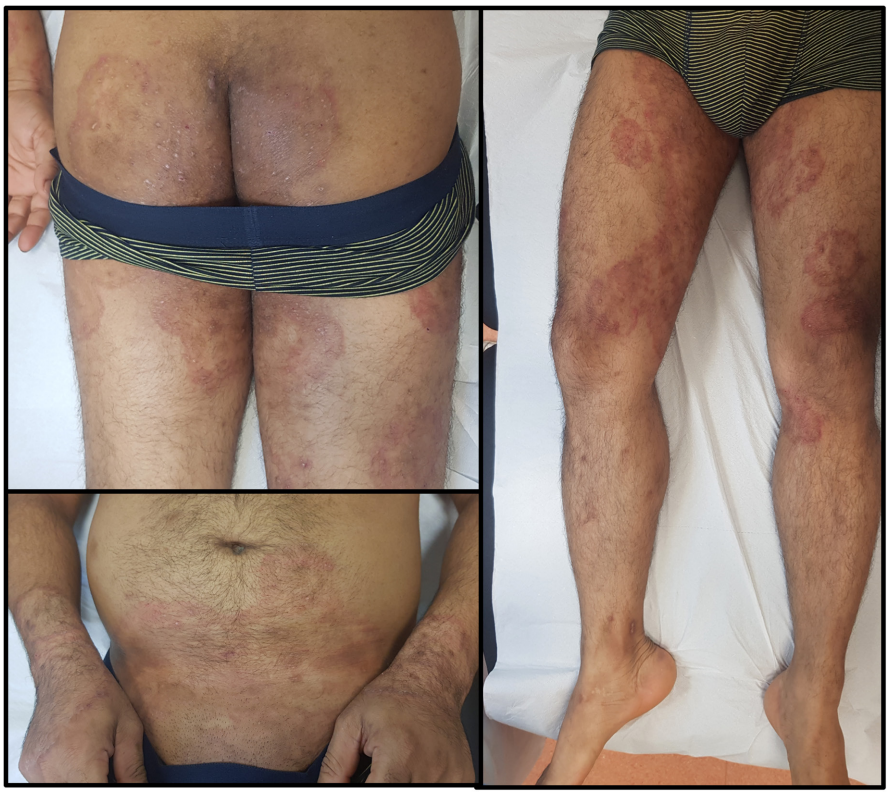

FIGURE 1 Widespread *tinea cruris* and *tinea corporis* presenting as scaly macules with a raised, defined margin involving different areas of the body despite 4 weeks treatment with systemic terbinafine 250/day.

mutation showed 100% homology with the sequence Genebank ID OL415221 as reported in Sabater et al.[8] The *T. indotineae* strain (IUM 22-0026) with the F415C mutation has been registered by us in Genbank (accession number OP883944).

All patients responded to itraconazole administered orally (200 mg/day for 14 days) without recurrence of the infection after 12 weeks from the beginning of therapy.

4 | DISCUSSION

The prevalence of dermatophytosis is continuously increasing, with an estimated prevalence of 20%–25% among the world population.[1] These infections are usually easily treatable with local or systemic antifungals. However, the numerous reports of resistance to terbinafine from all over the world are now arousing justified attention and concern, considering the limited number of antimycotics available. Dermatophytes isolates with reduced terbinafine susceptibility have been reported in the literature with a resistance rate ranging from 1% to 32%[4,14,17] depending on the species and the geographical area. Several factors have been implicated in the outbreak, including the uncontrolled over-the-counter sale of topical creams that contain combinations of steroids, antifungals and antibiotics.[5] The molecular mechanism of terbinafine resistance in *Trichophyton* spp. involves single point mutations in the SQLE gene, encoding the enzyme involved in ergosterol biosynthesis.[4,15,18] The inhibition of this enzyme leads to accumulation of squalene inside the fungal cells, depletion of ergosterol and finally causes cell death.

Our sequencing of the SQLE gene identified three previously described point mutations in the *T. rubrum* strain and in three *T. indotineae* strains that lead to missense mutation: L393F, F397L and L393S. We report a new missense mutations F415C in one *T. indotineae* strain, leading to terbinafine resistance. All of these amino acids are located in pivotal positions for the binding of terbinafine to the protein. For this reason, they are considered 'hot spots' and MIC values justified by their mutations are much higher than those caused by other point mutations described in literature.[11] In the study by Ditte et al. the SQLE profile showed that the majority of terbinafine resistance in the isolates was caused by F397L, leading to high terbinafine MICs (0.5–>4 mg/L). This is followed by L393F and L393S, which are also recognised causes of resistance in various countries, mainly in Asia and Europe.[11]

At this moment MALDI-TOF-MS is unable to correctly identify at species level *Trichophyton* isolates, and in particular *T. mentagrophytes* spp. complex. Only the ITS sequencing allows to discriminate the new species *T. indotineae* which is therefore definitely underestimated. We reported for the first time the presence in Italy of *T. indotineae*, a species that spread rapidly in India and surrounding regions with very high rates of resistance to terbinafine.[18] In recent years *T. indotineae* has been isolated from several European countries including Germany, Switzerland, Greece, Belgium, Germany, Finland, France and Denmark.[4,10,15,19–23] Macro and microscopic characteristics of *T. indotineae* colonies are identical to other *T. mentagrophytes* spp. Due to the inability of classical methods to differentiate it from other *T. mentagrophytes* spp., probably a larger number of countries participate in this epidemic compared to those listed in the literature. Currently *T. indotineae* is the predominant dermatophyte in

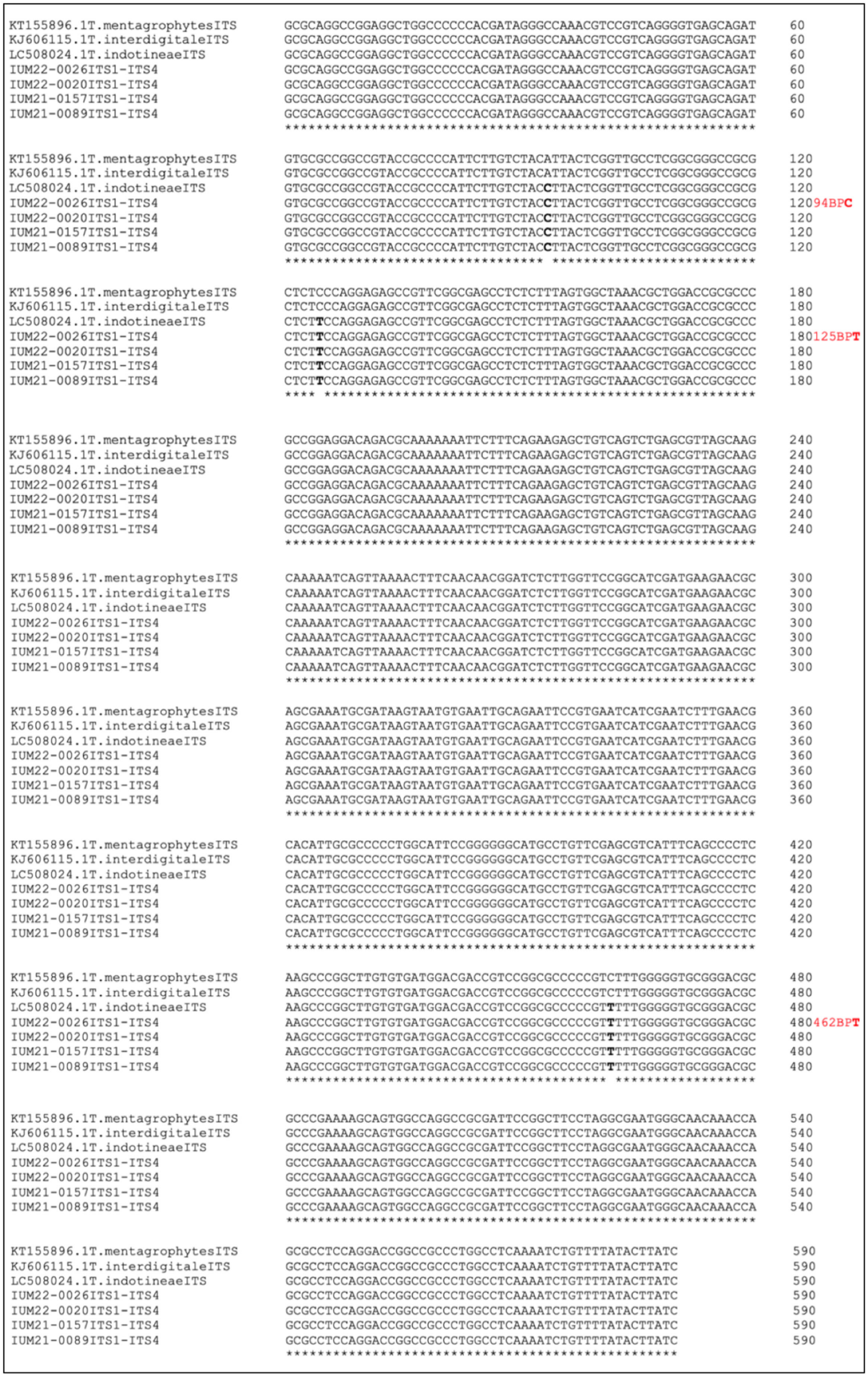

FIGURE 2 Comparison between the nucleotide sequences of the ITS1-5,8S-ITS2 region of the analysed isolates. Reference sequences: *T. indotineae* LC508024.1, *T. interdigitale* KJ606115.1, *T. mentagrophytes* KT155896.1. Bold letters indicate three single polymorphisms identity with the reference strain of *T. indotineae*. An asterisk indicates identity with the nucleotide found in the *Trichophyton* ITS sequence analysed.

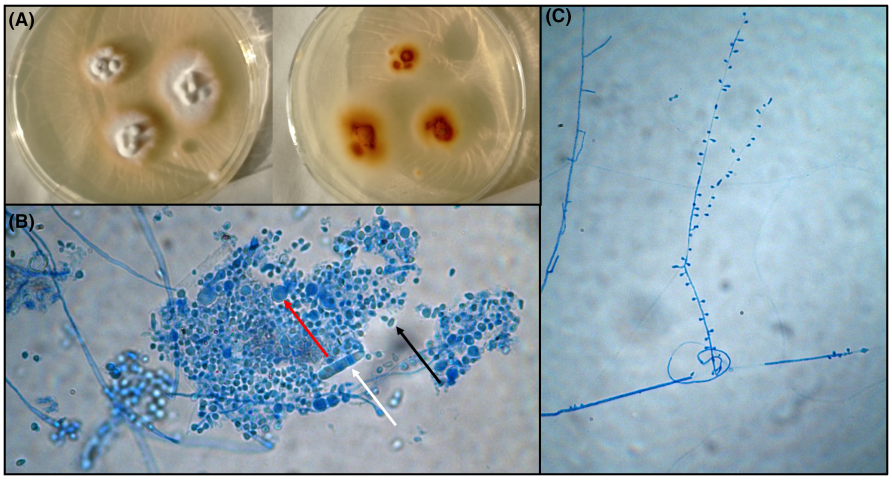

FIGURE 3 Macro and microscopic aspect of isolated *T. indotineae* strains: (A) colony, obverse and reverse; (B) microscopic elements: macroconidia (white arrow), microconidia (black arrow) and chlamydospores (red arrow); (C) microscopic aspect of the spirulina hypha and conidia on the hypha.

TABLE 1 MIC values (90% and 50% growth inhibition) of *T. rubrum* and *T. indotineae* strains for terbinafine and itraconazole with related missense mutations in SQLE enzyme.

	Terbinafine		Itraconazole		
Isolate	MIC (mg/L) 90% growth inhibition	MIC (mg/L) 50% growth inhibition	MIC (mg/L) 90% growth inhibition	MIC (mg/L) 50% growth inhibition	*SQLE* mutation
T. rubrum IUM 22-0002	4	0.25	0.125	0.06	L393F
T. indotineae IUM 22-0010	4	0.5	0.03	0.03	F397L
T. indotineae IUM 22-0011	1	0.5	0.06	0.03	F397L
T. indotineae IUM 22-0020	0.25	0.25	0.125	0.06	L393S
T. indotineae IUM 22-0026	0.5	0.5	0.125	0.06	F415C

Abbreviation: SQLE, squalene epoxidase.

India[24] and due to the high virulence of this species we cannot exclude that this epidemiologic shift will also occur in Europe. In the present study, *T. indotineae* was confirmed as the principal dermatophyte resistant to terbinafine with widespread infections as one of its typical clinical manifestations.

Dermatophyte antifungal susceptibility testing are not routinely performed due to cost, time and technical constraints. For this reason, terbinafine-resistant strains may be overlooked and underestimated. The rapid spread of resistance in *Trichophyton* spp. all over the world highlights the importance of an established and standardised procedure for performing AFST. The European Committee on Antimicrobial Susceptibility Testing Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST) has released in 2021, a method for AFST against microconidia-forming dermatophytes including tentative MIC ranges for quality control strains and tentative breakpoints against *T. rubrum*, *T. interdigitale* and *T. indotineae*.[13] Currently the EUCAST method is the only standardised method described step by step for the determination of the in vitro susceptibility of dermatophyte strains. In EUCAST method, the growth medium has been implemented with chloramphenicol and cycloheximide in order to reduce contaminations and to render the medium selective for dermatophytes. This permit to avoid interference with end-point reading and repetitions of the test forced by contaminants. Spectrophotometric reading of the endpoint was also added using 50% growth inhibition as a method to evaluate test results. The 50% endpoint is essential to standardise this type of test and explains how some studies using a 90% inhibition endpoint show much higher MICs and resistance incidences.[16] This method should help make terbinafine susceptibility testing more accessible. The EUCAST cut-offs proved to be particularly effective in identifying actual resistant species: most of the strains tested with MIC values considered resistant, later turned out to be non-wild-type for SQLE. The rare cases of wild-type SQLE with MICs above the EUCAST cut-off can be explained by other resistance mechanisms such as efflux pumps.[25] Kano et al. found that the expression levels of the *PDR1*, *MDR1*, *MDR2* and *MDR4* genes were 2–4 times higher

in the terbinafine-resistant strain grown in the presence of 0.14 mg/mL of terbinafine than in terbinafine-susceptible strains cultured in the absence of terbinafine.[26] According to previous studies, disruption of the *MDR2* gene leads mutants more susceptible to terbinafine than control strains, suggesting that one alternative to SQLE point mutation mechanisms of terbinafine resistance may involve efflux pumps.[25] Our case series confirm the reliability of EUCAST method and the ECOFF provided, with a 100% correlation observed between in vivo resistance and in vitro resistance.

In Italy susceptibility tests are reserved only in selected cases, but it cannot be excluded that these tests 1 day will be routinely proposed. Considering the extremely limited number of antifungals effective against dermatophytes and the increasing number of terbinafine-resistant *Trichophyton* isolates, laboratories must adopt in vitro AFST in order to monitor the emergence of resistance in the community. Moreover, prolonged therapy with antifungals in resistant strains expose the patients to possible side effects and could be a risk factor for selecting resistant isolates.

Italy and other European countries do not currently have surveillance programs for dermatophytosis resistant to terbinafine. The European medical community is watching unprepared to the explosive growth of this problem, continuously worsened by the increasing number of migrants coming from countries where this kind of resistance is already common.

5 | CONCLUSIONS

We report the first cases of terbinafine-resistant *Trichophyton* isolates in the Italian population. In Italy there are active surveillance programs for *Candida* and *Cryptococcus* resistance to systemic therapy with azoles, but currently there is no surveillance network for dermatophytosis. Solid antifungal management programs will be needed to promote more responsible use of these drugs and preserve their therapeutic efficacy to control antifungal resistance. Dermatologists should be aware of the possibility of infections caused by resistant dermatophytes, especially in widespread presentations or recalcitrant cases.

Furthermore, a new species in the *T. mentagrophytes* spp. complex is expanding globally; this species had previously been named *T. mentagrophytes genotype VIII* on the basis of the internal transcribed spacer sequence and then reclassified as *T. indotineae* due to clinical and genetic peculiarities. Our study demonstrates that this pathogen has arrived in Italy, probably imported by people from countries where this species is endemic. The inability of classical methods to differentiate it from *T. mentagrophytes* probably makes the prevalence of this species very underestimated.

AUTHOR CONTRIBUTIONS

P. Bortoluzzi: Conceptualization; investigation; writing – original draft; data curation. **A. Prigitano:** Investigation; methodology; data curation. **A. Sechi:** Conceptualization; investigation; validation. **V. Boneschi:** Conceptualization; investigation; methodology; writing – review and editing. **F. Germiniasi:** Investigation; validation. **M.C. Esposto:** Investigation; writing – review and editing; supervision. **L. Romanò:** Supervision; project administration; writing – review and editing. **G. Pavan:** Conceptualization; investigation; formal analysis. **C. Matinato:** Investigation; conceptualization; writing – review and editing; formal analysis. **S. Veraldi:** Writing – review and editing; supervision. **A.V. Marzano:** Supervision; writing – review and editing; validation; conceptualization. **A. Grancini:** Conceptualization; investigation; visualization; supervision; data curation.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The patients in this manuscript have given written informed consent to publication of their case details.

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