**ORIGINAL ARTICLE**

# Antifungal drug susceptibility profile of clinically important dermatophytes and determination of point mutations in terbinafine-resistant isolates

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## Abstract

With regard to increasing number of antifungal-resistant dermatophytes, antifungal susceptibility testing of dermatophytes serves as a useful tool in managing clinical dermatophytosis. This study aimed to determine antifungal susceptibility profile of clinically important dermatophytes and determination of point mutations in terbinafine-resistant isolates. Based on our results, dermatophytosis was confirmed in 97 cases by direct microscopic examination, culture, and sequencing of ITS region. Antifungal susceptibility of 97 dermatophyte isolates distributed in four species including *Trichophyton interdigitale* (26 isolates), *T. rubrum* (19 isolates), *T. tonsurans* (29 isolates), and *Epidermophyton floccosum* (21 isolates) was assessed to nine antifungal agents using CLSI M38-A2 guidelines. Minimum inhibitory concentration range (MIC range) for luliconazole and terbinafine was 0.001–0.008 μg/ml and 0.003–> 32 μg/ml, compared to 0.03–64 μg/ml for griseofulvin, 0.01–16 μg/ml for itraconazole and voriconazole, 0.03–8 μg/ml for ketoconazole, 0.03–32 μg/ml for econazole, 0.03–1 μg/ml for lanoconazole, and 0.01–4 μg/ml for butenafine. *Trichophyton tonsurans* was the most susceptible (MIC = 0.006 μg/ml) and *E. floccosum* was the most resistant (MIC = 0.02 μg/ml) species to terbinafine. Terbinafine resistance was reported for two species, i.e., *T. rubrum* and *T. tonsurans* at the total rate of 2% which was due to Leu393Phe substitution in both species. Taken together, our results assist clinicians and prompt the current knowledge about the necessity of antifungal susceptibility testing to select effective strategies for management of clinical cases of dermatophytosis.

**Keywords** Dermatophytes · Antifungal susceptibility testing · Dermatophytosis · Point mutation · Terbinafine resistance

## Introduction

Antifungal susceptibility testing of dermatophytes is a helpful tool for managing patients with different types of dermatophytosis [1]. It has been estimated that over 500 million $ per year spent for treatment of dermatophyte infections [2]. The increased use of antifungal drugs, often for prolonged periods, has led to the recognition of the phenomenon of

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acquired antifungal resistance [3]. A number of antifungal agents have been recommended for treating all types of dermatophytosis. Because of provides long-term clinical efficacy and lower relapse [4], terbinafine (TER) is considered as the best choice drug for treatment of dermatophytosis [5, 6]. Unfortunately, reports of drug-resistant strains to TER are increasing by the year [7–9]. Terbinafine is an allylamine antifungal drug that acts as an inhibitor of squalene epoxidase (*SQLE*), a crucial enzyme involved in ergosterol biosynthesis [10]. Inhibition of this enzyme leads to accumulation of squalene inside the fungal cells, depletion of ergosterol, and finally causes cell death [9, 11]. The molecular mechanisms of TER resistance in *T. rubrum* clinical strains have been investigated in different studies [8, 10, 12–15]. Furthermore, TER resistance has been reported in clinical *T. interdigitale* isolates [8, 15]. The resistance mechanism involves the single point mutations at one of four positions (Leu393, Phe397, Phe415, and His440) of the *SQLE* protein [8, 10, 12–15]. However, no effective mutations mediated by efflux transporter genes of

the ATP-binding cassette (ABC) or the major facilitator superfamily (MFS) transporter family have yet been reported in dermatophyte isolates [8]. Previous studies have focused on *T. rubrum* and *T. interdigitale* isolates resistant to TER [8, 10, 12–15]. In the present study, in addition to *T. rubrum*, point mutations of *T. tonsurans* and *E. floccosum* isolates resistant to TER were investigated. Likewise, in vitro antifungal activity of common and new antifungal drugs including TER, griseofulvin (GRI), itraconazole (ITC), voriconazole (VOC), luliconazole (LUL), lanoconazole (LAN), ketoconazole (KTC), butenafine (BUT), and econazole (ECO) was assessed against 97 dermatophyte isolates using CLSI broth microdilution M38-A2 method together with evaluating point mutations in strains resistant to TER for the first time in Iran.

## Materials and methods

### Chemical antifungal drugs

All tested antifungal drugs except terbinafine which was obtained from Dr. Reddy's Laboratories were purchased from Sigma-Aldrich (St. Louis, MO, USA). Compounds were dissolved in dimethylsulfoxide (Sigma-Aldrich®, St. Louis, USA) at a final concentration of 10 mg/ml.

### Clinical fungal isolates

Clinical samples obtained from patients suspected to dermatophytosis referred to the Department of Mycology of the Pasteur Institute of Iran were examined for etiologic dermatophytes. Identification of isolates was primarily carried out using direct microscopy and culture and confirmed by ITS sequencing. A total 97 identified dermatophyte isolates including *T. rubrum* ($n = 19$), *T. interdigitale* ($n = 26$), *T. tonsurans* ($n = 29$), and *E. floccosum* ($n = 23$) were included in this study. Reference strains of *T. rubrum* (PFCC 51431) and *T. mentagrophytes* (PTCC 5054) were tested in all steps. The datasets generated during the current study are available in the Pathogenic Fungi Culture Collection repository, http://fa.pasteur.ac.ir/MBankResult.aspx.

### Molecular identification by PCR assay and sequencing targeting ITS region

All dermatophyte strains were cultured on mycobiotic agar (Merck, Germany) and incubated at 28 °C for 7 days. DNA was extracted using the chloroform and proteinase K method. The fungal mycelium was disrupted by Pestle in presence of liquid nitrogen and DNA extraction buffer (200 M Tris-HCl, pH 8, 25 mM EDTA, SDS 0.5% *W/V*, NaCl 250 mM). After that, the mixture was vortexed, proteinase K was added, and total content was incubated at 55 °C for 60 min. The DNA was extracted with an equal volume of chloroform. Total nucleic acids were precipitated with isopropanol, washed in 300 µl ethanol 70%, air-dried, rehydrated in 50 µl TE buffer, and stored at − 20 °C till it was used [16].

The ITS region was PCR amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCT CCGCTTATTGATATGC-3′) [17]. Each mixture contained 12.5 µl of Premix, 1 µl of DNA template, 0.3 µM of each primer, and enough water to reach a final reaction volume of 25 µl. Negative controls (water instead of fungal DNA) were added to each PCR. The reaction mixture was initially denatured at 95 °C for 5 min followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 45 s at 72 °C, and a terminal extension step of 72 °C for 5 min. Five microliters of the PCR products was electrophoresed on 1% agarose gel in TAE buffer and then observed and photographed under ultraviolet irradiation.

### Antifungal drug susceptibility testing

Stock solutions of antifungal drugs were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) according to CLSI M38-A2 broth microdilution protocol [18] and diluted in standard RPMI 1640 medium buffered to pH 7.0 with 0.165 mol/l 3-(*N*-morpholino)propanesulfonic acid (MOPS) with L-glutamine without bicarbonate (Sigma-Aldrich, St. Louis, MO, USA). The final concentration of drugs, fungal spore suspensions, and drug dilutions was prepared according to CLSI M38-A2 broth microdilution protocol [18]. All the dermatophytes were exposed to different concentrations of antifungal drugs in 96-well round bottom microplates. The inoculated microplates were incubated at 35 °C and visually assessed for fungal growth after 4 days incubation. The MIC was defined as the point at which the growth of dermatophyte was inhibited by 80% for eight antifungals in comparison with the control. All tests were performed in duplicate. *Trichophyton rubrum* (PTCC 5143) and *C. parapsilosis* (ATCC 22019) were used as quality controls. MIC range, geometric mean, $MIC_{50}$, and $MIC_{90}$ were provided for all the isolates tested.

### PCR assay targeting the *SQLE* region

For *SQLE* gene amplification, a novel set of pan-dermatophyte primers was designed manually with lasergene 7 software as Drsq1 (5′-TTGCCAACGGAGGTGTAAAG-3′) and Drsq2 (5′-GGGGCCATCTATAAGTCCAAGTT-3′). This primer designed by use of DNA sequence of clinical *T. rubrum* isolated NFI 5166 [13]. The PCR was performed using a Taq DNA Polymerase Master Mix, with primers Drsq1/2. Each mixture contained 25 µl of Premix 3 µl of DNA template, 0.6 µM of each primer, and enough water to reach a final reaction volume of 50 µl. Negative controls were added to each PCR. The reaction mixture was initially

denatured at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C, and 45 s at 72 °C, and a terminal extension step of 72 °C for 5 min. Five microliters of the PCR products (520 bp) was electrophoresed on 1% agarose gel in TAE buffer and then observed and photographed under ultraviolet irradiation.

### ITS and *SQLE* sequencing

The PCR products of ITS and *SQLE* regions were sequenced by the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit. The forward and reverse sequences of isolates showing reduced susceptibility to TER were subjected to ClustalW pairwise alignment using the MEGA7.0.21 software and edited manually to improve the alignment accuracy [10].

### Statistical analysis

Quantitative data of MIC range, geometric mean MIC, $MIC_{50}$, and $MIC_{90}$ were subjected to statistical analysis of one-way ANOVA and multiple comparisons test using the statistical SPSS package version 19. *P* values of < 0.05 were considered significant.

## Results

### Clinical features of studied dermatophytes

General features of 97 dermatophyte isolates studied in the present work are summarized in Table S1. Among the 97 patients, 77.3% were male and 22.7% were female distributed in the age range of 2 to 77 years. The most common infected age group was the 21 to 30 years (Fig. 1A). Tinea pedis (29.8%) and tinea cruris (29.8%) were the most common types of dermatophytosis followed by tinea corporis (18.5%), tinea capitis (9.2%), tinea faciei (6.1%), and tinea manuum (2%). The difference between the prevalence of tinea pedis in men and women was statistically significant and the most common species involved was *T. interdigitale*. Also, *E. floccosum* in tinea cruris was the most frequent fungal pathogen, followed by *T. tonsurans* in other anatomical sources. Overall, *T. tonsurans* was the most important dermatophyte regard to infecting various anatomical sites (Fig. 1B).

### Identification of *dermatophytes* by ITS-PCR sequencing

All dermatophyte isolates further identified by ITS-rDNA region sequencing. The query sequences were paired with those in the GenBank database by the Blast analysis. The sequences were deposited in the GenBank ITS-rDNA and the nucleotide sequences of the following accession numbers were considered: *T. interdigitale*: MF109036-MF109062, *T. rubrum*: MF155577-MF155596, *T. tonsurans*: MH337848, MF158259-MF158287, and *E. floccosum*: MF158288-MF158310.

### Antifungal susceptibility of dermatophyte isolates

The MIC range, geometric mean MIC, $MIC_{50}$, and $MIC_{90}$ were obtained by the CLSI method for the dermatophyte species against the eight antifungals tested (Table 1). LUL susceptibility was tested against isolates resistant to other drugs (Table 2). A significant sensitivity to TER was reported in *T. tonsurans* ($MIC_{50} = 0.006$ µg/ml). The most susceptible and resistant species to GRI were *T. interdigitale* ($MIC_{50} = 0.12$ µg/ml) and *E. floccosum* ($MIC_{50} = 1.0$ µg/ml), respectively. TER and GRI had the lowest and the highest geometric mean MICs which were 0.01 and 1.64 µg/ml for *T. interdigitale* and *E. floccosum*, respectively. TER was the most effective antifungal drug against all dermatophyte species (Table 1). Among 99 tested isolates, 5 (2 isolates of *T. rubrum*, 2 isolates of *T. tonsurans*, and 1 isolate of *E. floccosum*) showed reduced TER susceptibility.

### Point mutations detected in the *SQLE* gene of terbinafine-resistant isolates

After amplification of the genomic DNAs of five isolates that showed reduced susceptibility to TER by the PCR using Drsq, the expected size (520 bp) of the product for each species was generated in all cases. The forward and reverse sequences of each sample were subjected to ClustalW pairwise alignment using the MEGA7.0.21 software and edited manually to improve the alignment accuracy (Fig. S1). The data were compared in GenBank using the BLASTn. Then, the nucleic acid sequence converts to the amino acid sequence, the alignment of these amino acid is presented in Fig. S2. Among 5 isolates which showed reduced susceptibility to terbinafine, only 2 isolates (89_610, 92_256) with MICs > 32 showed amino acid substitution at position (Leu393Phe) of the squalene epoxidase protein. In both of these isolates were found A- to -T substitutions at position 1179 of the squalene epoxidase partial sequence gene, leading to the replacement of 393 by Phe in the squalene epoxidase protein. The DNA sequences of two TER resistant isolates have been deposited in the GenBank with the accession numbers of MH523150 for *T. rubrum* and MH523151 for *T. tonsurans*.

## Discussion

In the present study, 97 isolates of dermatophytes isolated from clinical cases of dermatophytosis were identified at

**Fig. 1** Distribution of dermatophytosis according to the age (**a**) and dermatophyte species involved (**b**)
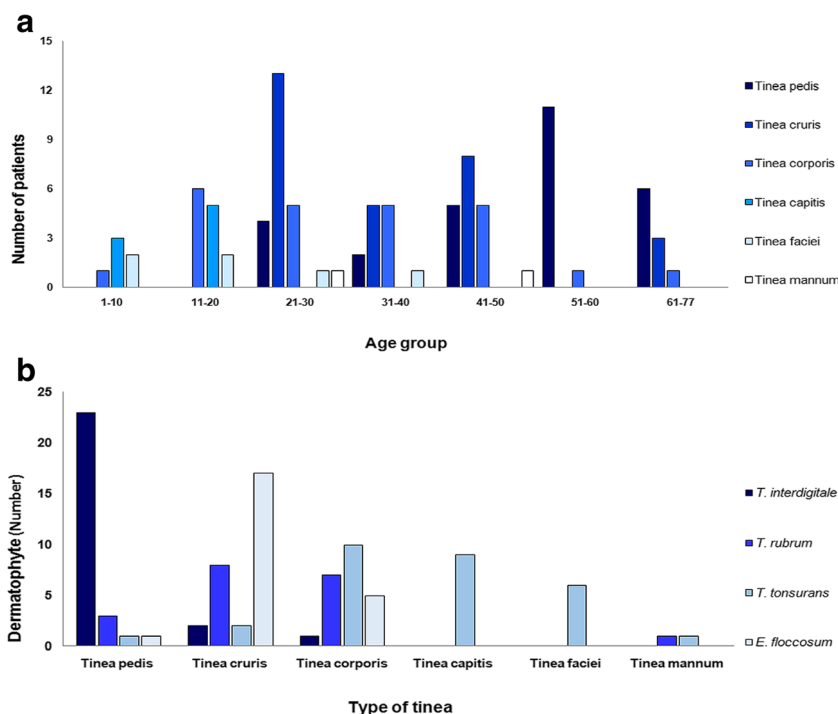


species level and subjected to antifungal susceptibility testing to nine antifungal drugs and resistance mechanisms to terbinafine. Besides the global importance of dermatophytosis as the commonest superficial infection in the world, in vitro susceptibility testing plays an essential role to determine emerging resistance patterns among etiologic dermatophytes. Generally, TER therapy is considered as common therapeutic strategy for curing clinical dermatophytosis. In a few studies, the point mutation in *T. rubrum* and *T. interdigitale* isolates resistant to TER has been investigated. In this study, in addition the point mutation of *T. rubrum* isolates resistant to terbinafine, for the first time point mutations of *T. tonsurans* and *E. floccosum* isolates that showed reduced TER susceptibility were investigated. According to the previous studies, relatively low levels of TER resistance were recorded in dermatophyte strains, proving that this drug encompasses suitable activity on a variety of clinical strains. In the present study, TER showed a variable range of activity against the different species of *Trichophyton* (MICs of 0.003–2) and *Epidermophyton* (MICs of 0.003–1). MICs of ITC and VOC for *Trichophyton* and *Epidermophyton* species were almost equal, while MICs of KTC, ECO, LAN, and BUT for *T. rubrum* species were higher than the other species. Our results highlight the usefulness of TER in majority cases of dermatophytosis especially in infections caused by GRI resistance species. This finding is in accordance with previous investigations [9, 19].

The $MIC_{50}$ for LUL was 0.004 μg/ml against isolates which were resistant to other drugs while it was 0.01 μg/ml for terbinafine against all tested isolates. Interestingly, all isolates resistant to tested antifungal drugs even those resistant to terbinafine were susceptible to luliconazole. This is in accordance with the reports of Wiederhold et al. [20] and Baghi et al. [21].

In the present study, the Gm MIC of TER was lower for *T. interdigitale* and *T. tonsurans* and higher for *T. rubrum* and *E. floccosum* than that those reported by Ansari et al. [22]. Also, the Gm MIC of ITC for *T. rubrum*, *T. interdigitale*, and *T. tonsurans* was lower than that of Esteban et al. [23] and Adimi et al. [24].

According to our results, the MIC of GRI against 99 dermatophyte isolates was in the range of 0.03 to 64 μg/ml. *T. interdigitale* was the most susceptible species to GRI ($MIC_{50}$, 0.12 μg/ml). GRI showed the reduced susceptibility to *E. floccosum* ($MIC_{50}$, 1 μg/ml). These results are in agreement with the report by Nowrozi et al. [25].

It has been shown that the resistance mechanism of dermatophytes for TER is related to the single point mutations at one of four positions (Leu393, Phe397, Phe415, and His440) of the *SQLE* protein [8, 10, 12–15]. Recently, Yamada et al. [8] reported a TER resistant rate of 1% which mainly included *T. rubrum* isolates. In the present study among 97 tested isolates, 5 (5%) showed reduced terbinafine susceptibility, of which 2 were found to be related to amino acid substitution Leu393 by Phe in the squalene epoxidase protein. This is in accordance with the reports by other investigators [8, 10, 15]. Singh et al. [15] reported the substitution Phe397Leu and Leu393Phe in TER resistant *T. interdigitale* isolates, while Yamada et al. [8] showed the substitution in Phe397Leu as the resistance mechanism to TER. We did not find resistance to TER in 27 tested *T. interdigitale* isolates.

**Table 1** In vitro antifungal susceptibility of 99 dermatophyte strains (97 clinical and two reference isolates) against eight antifungal agents

| Dermatophyte species | Antifungal drug | MIC (μg/ml) | | | |
|---|---|---|---|---|---|
| | | Range | 50 | 90 | G mean |
| T. interdigitale | TER | 0.003–0.125 | 0.01 | 0.06 | 0.01 |
| (n = 27) | GRI | 0.03–64 | 0.12 | 35.2 | 0.41 |
| | ITC | 0.01–4 | 0.06 | 1.3 | 0.07 |
| | VOC | 0.01–16 | 0.37 | 8.8 | 0.41 |
| | KTC | 0.03–4 | 0.25 | 2.2 | 0.32 |
| | ECO | 0.03–0.5 | 0.06 | 0.5 | 0.08 |
| | LAN | 0.03–0.5 | 0.06 | 0.5 | 0.09 |
| | BUT | 0.03–0.5 | 0.06 | 0.5 | 0.09 |
| T. rubrum | TER | 0.003–>32 | 0.03 | 1.3 | 0.04 |
| (n = 20) | GRI | 0.06–64 | 0.25 | 57.6 | 0.66 |
| | ITC | 0.01–8 | 0.03 | 3.25 | 0.05 |
| | VOC | 0.01–16 | 0.5 | 14.4 | 0.60 |
| | KTC | 0.06–8 | 0.12 | 3.6 | 0.28 |
| | ECO | 0.03–32 | 0.06 | 0.5 | 0.10 |
| | LAN | 0.03–1 | 0.25 | 1 | 0.24 |
| | BUT | 0.03–4 | 0.12 | 0.9 | 0.17 |
| T. tonsurans | TER | 0.003–>32 | 0.006 | 0.25 | 0.01 |
| (n = 29) | GRI | 0.03–64 | 0.25 | 32 | 0.46 |
| | ITC | 0.01–16 | 0.03 | 4 | 0.07 |
| | VOC | 0.01–16 | 0.125 | 4 | 0.13 |
| | KTC | 0.03–2 | 0.125 | 1 | 0.16 |
| | ECO | 0.03–0.5 | 0.06 | 0.5 | 0.07 |
| | LAN | 0.03–0.5 | 0.25 | 0.5 | 0.17 |
| | BUT | 0.01–0.5 | 0.06 | 0.5 | 0.07 |
| E. floccosum | TER | 0.003–1 | 0.02 | 0.1875 | 0.02 |
| (n = 23) | GRI | 0.03–64 | 1 | 64 | 1.64 |
| | ITC | 0.01–16 | 0.03 | 12 | 0.08 |
| | VOC | 0.03–16 | 0.25 | 12 | 0.32 |
| | KTC | 0.03–2 | 0.06 | 2 | 0.11 |
| | ECO | 0.03–0.25 | 0.03 | 0.25 | 0.05 |
| | LAN | 0.03–0.5 | 0.25 | 0.5 | 0.20 |
| | BUT | 0.01–0.25 | 0.03 | 0.25 | 0.04 |

*MIC* minimal inhibitory concentration, *G mean* geometric mean, *TER* Terbinafine, *GRI* Griseofulvin, *ITC* Itraconazole, *VOC* Voriconazole, *LAN* Lanoconazole, *KTC* Ketoconazole, *BUT* Butenafine, *ECO* Econazole

**Table 2** Minimum inhibitory concentrations of luliconazole for dermatophyte isolates which were resistant to at least one of other eight tested antifungal drugs

| Dermatophyte species | MIC (μg/ml) | | |
|---|---|---|---|
| | Range | 50 | 90 |
| T. interdigitale (n = 6) | 0.001–0.004 | 0.002 | 0.002 |
| T. rubrum (n = 7) | 0.002–0.008 | 0.004 | 0.004 |
| T. tonsurans (n = 5) | 0.002–0.008 | 0.004 | 0.004 |
| E. floccosum (n = 11) | 0.001–0.008 | 0.003 | 0.003 |

MIC, minimal inhibitory concentration

Strain resistance to TER has been reported for dermatophytes isolated from tinea pedis and tinea unguium [8, 15]. We reported TER resistance for dermatophytes isolated from tinea pedis and tinea corporis. Interestingly, in the study by Singh et al. [15], of 20 patients infected with TER resistant *T. interdigitale* isolates, 10 did not respond to TER therapy. Also, Yamada et al. [8] reported that 8 of the 17 patients with TER resistant etiologic dermatophytes were resistant to TER therapy.

Taken together, results of the present study further indicate the importance of dermatophytes as fungal pathogens of the skin and nail and confirms the necessity of antifungal susceptibility testing to choose effective drugs and efficient

management strategies of clinical case of dermatophytosis. Likewise, increasing resistance to TER as the choice drug for treatment of dermatophytosis even in new species like as *T. rubrum* and *T. tonsurans* reported by us in the present shows the urgent of caring with the use of antifungal drugs at the clinic and stressed the necessity of follow up the patient's condition after antifungal treatment.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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