Rapid Molecular Detection of Terbinafine-resistant Dermatophytes

Rui Kano¹, Hiromitsu Noguchi²,³, Kazutoshi Harada⁴, and Masataro Hiruma³

¹ Department of Veterinary Dermatology, Nihon University College of Bioresource Sciences
² Noguchi Dermatology Clinic
³ Ochanomizu Institute for Medical Mycology and Allergology
⁴ Department of Dermatology, Tokyo Medical University

ABSTRACT

Terbinafine (TRF)-resistant Trichophyton interdigitale and Trichophyton rubrum have been isolated from human patients. These strains have missense mutations (Leu393Ser/Phe or Phe397Leu) in the squalene epoxidase-encoding gene, SQLE. We developed a PCR detection method to identify hotspot mutation sites in SQLE genes of dermatophytes. To sequence hotspots in isolates, we prepared primers based on conserved sequences of T. rubrum and T. interdigitale SQLEs. Approximately 390-bp long DNA bands for T. rubrum, T. interdigitale, and Trichophyton indotineae strains were sequenced. Hotspots were detected only in TRF-resistant strains. This PCR-based method is simpler and more rapid than the conventional test.

Key words: dermatophytosis, polymerase chain reaction, resistance, terbinafine, Trichophyton rubrum

Introduction

Trichophyton interdigitale and Trichophyton rubrum are anthropophilic species that are most frequently isolated from tinea unguium, tinea pedis, and tinea corporis worldwide.²⁻⁵ Terbinafine (TRF) has been used in the treatment of these infections for more than 20 years. Recently, TRF-resistant T. interdigitale and T. rubrum were isolated from human patients.²⁻⁴ These strains have missense mutations (Leu393Ser/Phe or Phe397Leu) in the squalene epoxidase-encoding gene, SQLE.²⁻⁴ These hotspot mutations may lead to a failure to block the ergosterol biosynthesis pathway by TRF.⁷

Trichophyton indotineae, a species newly designated in 2020 independent of T. interdigitale,² comprises highly TRF-resistant dermatophytes that are epidemic in North India. T. indotineae harbors a missense mutation (Phe397Leu) in SQLE.²⁻⁴ This high level of TRF resistance [minimum inhibitory concentration (MIC): > 32 µg/mL] in Indian dermatophyte isolates seems to be driving an ongoing outbreak of dermatophytoses in countries other than India.²⁻⁴

Antifungal susceptibility testing such as the Clinical and Laboratory Standards Institute (CLSI) protocol M38-A2 microdilution technique has been performed on clinical isolates to detect drug-resistant strains as a conventional method.²⁻⁴ However, it takes at least 4 weeks including pure culture on potato dextrose agar and incubation in 96-well plates to obtain results. Therefore, it is necessary to detect drug-resistant strains using a simple and quick method. In the present study, we improved the rapid and simple molecular detection method for Leu393Ser/Phe or Phe397Leu in the SQLE of dermatophytes. The clinical isolates examined in this study are listed in Table 1. Five T. interdigitale and five T. rubrum clinical isolates were obtained from ten human cases of tinea unguium and tinea pedis in Kumamoto, Japan, in 2020. One clinical isolate of T. indotineae (NUBS20020) was obtained from a human case of tinea corporis in Saitama, Japan, in 2020.

Two TRF-resistant strains of T. indotineae (NUBS19006 and NUBS19007) harbor a missense mutation (Phe397Leu) in SQLE.²⁻⁴ Two TRF-resistant strains of T. rubrum (N74 and N79) were obtained from two human cases of tinea unguium in Kumamoto, Japan.
To assess TRF susceptibility in these isolates, we performed the broth microdilution assay based on the CLSI M38-A2 guidelines with modifications as previously described\textsuperscript{13, 14).}

TRF MICs for the five \textit{T. interdigitale} isolates (N62, N63, N91, N93, and N94) and five \textit{T. rubrum} isolates (N64, N65, N66, N88, and N92) were <0.03-0.125 µg/mL (Table 1). TRF MIC for the \textit{T. indotineae} isolate (NUBS20020) was < 0.03 µg/mL (Table 1).

Table 1. Strains, TRF MICs (µg/mL), and mutation sites in \textit{SQLE} genes

| Strain                  | TRF MICs (µg/mL) | Nucleotide substitution\textsuperscript{1} | Amino acid substitution\textsuperscript{2} |
|-------------------------|-----------------|------------------------------------------|----------------------------------------|
| \textit{T. indotineae} NUBS19006 | >32\textsuperscript{3} | 3546C → A | F397L |
| \textit{T. indotineae} NUBS19007 | >32\textsuperscript{3} | 3546C → A | F397L |
| \textit{T. indotineae} NUBS20020 | <0.03 | ND |
| \textit{T. rubrum} N74        | >32\textsuperscript{4} | 1179A → T | L393F |
| \textit{T. rubrum} N79        | 32\textsuperscript{4}  | 1179A → T | L393F |
| \textit{T. interdigitale} N62 | <0.03 | ND |
| \textit{T. interdigitale} N63 | <0.03 | ND |
| \textit{T. interdigitale} N91 | <0.03 | ND |
| \textit{T. interdigitale} N94 | <0.03 | ND |
| \textit{T. rubrum} N64        | <0.03 | ND |
| \textit{T. rubrum} N65        | <0.03 | ND |
| \textit{T. rubrum} N66        | 0.125 | ND |
| \textit{T. rubrum} N88        | <0.03 | ND |
| \textit{T. rubrum} N92        | <0.03 | ND |

MIC: minimum inhibitory concentration; ND: not detected; TRF: terbinafine.

\textsuperscript{1}TRF MIC was reported in reference 8.

\textsuperscript{2}TRF MIC was reported in reference 15.

\textsuperscript{3}Nucleotide and amino acid sequences were compared to \textit{T. mentagrophytes} \textit{SQLE} (GenBank accession no. KU242352) or \textit{Trichophyton rubrum} CBS 118892 \textit{SQLE} (GenBank accession number, XM_003233797).

An approximately 390-bp long DNA band for each strain was excised from the gel (Fig. 2), purified using the ExoSAP-IT® kit (USB Corporation, Cleveland, OH, USA), and sequenced on an ABI PRISM 3130 DNA Analyzer (Thermo Fisher Scientific, Inc., Tokyo, Japan).

Comparative sequence analyses were carried out using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website.

In \textit{SQLE}, TRF-resistant strains of \textit{T. indotineae} (NUBS19006 and NUBS19007) harbored a single polymorphism (SNP) at position 3546 (C → A), which encoded Leu at position 393 (C → A) of the \textit{SQLE} gene.

Genomic DNA (100-200 ng) from clinical isolates was amplified by PCR in a volume of 30 µL, using a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 0.001% gelatin, 200 mM of each deoxynucleoside triphosphate, 1.0 U Taq polymerase (Takara, Kyoto, Japan), and 50 5M of primer pairs. Thirty cycles of PCR amplification were performed with the following conditions: denaturation for 30 s at 95°C, primer annealing for 30 s at 56°C, and extension for 1 min at 72°C. The resulting amplified DNA fragments were electrophoresed on a 2% (w/v) agarose gel with 1× TAE buffer and visualized by ethidium bromide staining.

Fig. 1. Nucleotide and amino acid substitutions of the mutation hotspot sites (Leu393Ser/Phe or Phe397Leu) in \textit{SQLE}.

A: Nucleotide and amino acid sequences were compared with \textit{T. mentagrophytes} \textit{SQLE} (GenBank accession no. KU242352).

B: Nucleotide and amino acid sequences were compared with \textit{Trichophyton rubrum} CBS 118892 \textit{SQLE} (GenBank accession number, XM_003233797). Bold characters and underlines indicate the nucleotide or amino acid substitutions.
codon 397 instead of Phe (Phe397Leu) in the *T. mentagrophytes* strain TIMM2789 (GenBank accession number, KU242352) (Table 1). TRF-resistant strains of *T. rubrum* (N74 and N79) harbored an SNP at position 1179 (A → T), which encoded Phe at codon 393 instead of Leu (L393F) in comparison with *T. rubrum* CBS 118892 squalene epoxidase (TERG_05717) mRNA, complete cds (GenBank accession number, XM_003233797) (Table 1).

All TRF-susceptible strains of *T. interdigitale* (N62, N63, N91, N93, and N94), *T. rubrum* (N64, N65, N66, N88, and N92) and *T. indotineae* (NUBS20020) did not have a mutation at the positions mentioned above (Table 1).

In this study, we developed a PCR detection method for hotspot mutation sites of Leu393Ser/Phe or Phe397Leu in the SQLE of dermatophytes. This method is simpler and more rapid than conventional tests, such as the *in vitro* antifungal susceptibility test. The *in vitro* antifungal susceptibility test requires at least 2 weeks to obtain results. Cultures are incubated for more than 7 days until good conidiation or sporulation is obtained, and another 5 days is needed for determining MICs for dermatophytes.

Dermatophytosis or tinea is predominant in about 20-25% of the world’s population\(^1\). TRF is one of the most therapeutic choices, since it is available as both systemic and topical drugs.

Yamada et al. recently reported that in Swiss patients analyzed in 2017, 1% (16/1644) of *T. rubrum* and 0.2% (1/412) of *T. interdigitale* strains demonstrated TRF resistance\(^1\). We expect that the prevalence of foot dermatophytosis due to antifungal drug-resistant strains will increase in Japanese patients. This study suggests that the molecular detection method is important in cases of treatment failure and that TRF resistance may be emerging in *T. rubrum* isolates in Japan. Dermatologists should be cautious about the prevalence of dermatophytosis due to antifungal drug-resistant strains.

**Conflicts of interest**

The authors are applying for the patent royalty (2021-002373) in Japan on the results of this study.

**References**

1) Kwon-Chung KJ, Bennett EJ: Dermatophytoses. In Medical Mycology. pp. 105-161 and 816-826. Lea & Febiger, Philadelphia, 1992.

2) Reiss E, Shadomy HJ, Lyon IIIGM: Dermatophytosis. In Fundamental Medical Mycology. pp. 527-566. Wiley-Blackwell, New Jersey, 2012.

3) Osborne CS, Leitner I, Favre B, Ryder NS: Amino acid substitution in *Trichophyton rubrum* squalene epoxidase associated with resistance to terbinafine. Antimicrob Agents Chemother 49: 2840-2844, 2005.

4) Yamada T, Maeda M, Alshahni MM, Tanaka R, Yaguchi T, Bontems O, Salamin K, Fratti M, Monod M: Terbinafine resistance of *Trichophyton* clinical isolates caused by specific point mutations in the squalene epoxidase gene. Antimicrob Agents Chemother 61: e00115-17, 2017.

5) Saunte DML, Hare RK, Jørgensen KM, Jørgensen R, Deleuran M, Zachariae CO, Thomsen SF, Bjørnskov-Halkier L, Kofoed K, Arendrup MC: Emerging terbinafine resistance in *Trichophyton*: clinical characteristics, squalene epoxidase gene mutations, and a reliable EUCAST method for detection. Antimicrob Agents Chemother 63: e01126-19, 2019.

6) Noguchi H, Matsumoto T, Hiruma M, Kimura U, Kano R, Yaguchi T, Fukushima S, Ihn H: Tinea unguium caused by terbinafine-resistant *Trichophyton rubrum* successfully treated with fosravuconazole. J Dermatol 46: e446-e447, 2019.

7) Rudramurthy SM, Shankarnarayan SA, Dogra S, Shaw D, Mustqat K, Paul RA, Naran T, Chakraborti A: Mutation in the squalene epoxidase gene of *Trichophyton interdigitale* and *Trichophyton rubrum* associated with allylamine resistance. Antimicrob Agents Chemother 62: e02522-17, 2018.

8) Kano R, Kimura U, Kakurai M, Hiruma J, Kamata H, Suga Y, Harada K: *Trichophyton indotineae* sp. nov.: A new highly terbinafine-resistant anthropophilic dermatophyte species. Mycopathologia 185: 947-958, 2020.
9) Singh A, Masih A, Khurana A, Singh PK, Gupta M, Hagen F, Meis JF, Chowdhary A: High terbinafine resistance in *Trichophyton interdigitale* isolates in Delhi, India harbouring mutations in the squalene epoxidase gene. *Mycoses* 61: 477-484, 2018.

10) Singh A, Masih A, Monroy-Nieto J, Singh PK, Bowers J, Travis J, Khurana A, Engelthaler DM, Meis JF, Chowdhary A: A unique multidrug-resistant clonal *Trichophyton* population distinct from *Trichophyton mentagrophytes/Trichophyton interdigitale* complex causing an ongoing alarming dermatophytosis outbreak in India: Genomic insights and resistance profile. *Fungal Genet Biol* 133: 103266, 2019. doi: 10.1016/j.fgb.2019.103266.

11) Saunte DML, Hare RK, Jørgensen KM, Jørgensen R, Deleuran M, Zachariae CO, Thomsen SF, Bjørnskov-Halkier L, Kofoed K, Arendrup MC: Emerging terbinafine resistance in *Trichophyton*: clinical characteristics, squalene epoxidase gene mutations, and a reliable EUCAST method for detection. *Antimicrob Agents Chemother* 63: e01126-19, 2019.

12) Friedman DZP, Schwartz IS: Emerging fungal infections: New patients, new patterns, and new pathogens. *J Fungi (Basel)* 5: 67, 2019.

13) CLSI document M38-A2: Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard, 2nd edn. Clinical Laboratory Standards Institute, Philadelphia, 2008.

14) Itoi S, Kano R, Hasegawa A, Kamata H: *In vitro* activities of antifungal agents against clinical isolates of dermatophytes from animals. *J Vet Med Sci* 74: 1067-1069, 2012.

15) Hiruma J, Noguchi H, Hase M, Tokuhisa Y, Shimizu T, Ogawa T, Hiruma M, Harada K, Kano R: Epidemiological study of terbinafine-resistant dermatophytes isolated from Japanese patients. *J Dermatol* 48: 564-567, 2021.

16) AL-Khikaoi FHO: Dermatophytosis a worldwide contiguous fungi infection: Growing challenge and few solutions. *Biomed Biotechnol Res J* 4: 117-122, 2020.