

PCR Typing of *Trichophyton Rubrum* Isolates by Specific Amplification of Subrepeat Elements in Ribosomal DNA Nontranscribed Spacer

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Abstract

Background: *Trichophyton rubrum* (*T. rubrum*) is the most common cause of dermatophytosis of skin and nail tissue. Strain identification in *Trichophyton rubrum* is important for identification of strain-related differences in infectivity potential or transmissibility and epidemiological studies. PCR typing could determine whether the original isolate is responsible for re-infection or a new strain has been acquired.

Methods: A minipreparation method for DNA from dermatophytes was used. Tandemly repetitive subelements (TRS-1 & TRS-2) of NTS region at ribosomal DNA of 23 *T. rubrum* isolates were amplified and the PCR products were separated by electrophoresis in 2% agarose gel (200 mA, 140 V), visualized by staining with ethidium bromide, and photographed.

Results: On the basis of copy number of TRS-1 and TRS-2, 8 out of our 23 samples were type 2 & II, respectively. Six of them were type 3 & II, four isolates were type 1 & II, two isolates were type 4 & II, two isolates were type 1 & I and one isolate was type 5 & II.

Conclusion: In this study, most of *T. rubrum* isolates were type 2 & II, dissimilar to European studies where type 1 & II has been the most common. The present study showed that 26.1% of Iranian isolates were type 1 in contrast with a previous study which has demonstrated a much lower prevalence in Asians (5%).

Keywords: *trichophyton rubrum*, PCR typing, dermatophytosis

Introduction

The dermatophytes are keratinophilic fungi belonging to three genera, *Trichophyton*, *Microsporum*, and *Epidermophyton*. The anthropophilic species *Trichophyton rubrum* (*T. rubrum*) is an important etiologic agent of tinea (ringworm) infections, including onychomycosis (nail infection), tinea cruris (groin infection), and tinea pedis (athlete's foot)¹. Lack of adequate methods for strain identification in *T. rubrum* has impeded efforts to identify strain-related differences in infectivity potential or transmissibility. A typing system would be useful to determine, for example, whether certain strains are more likely to cause

onychomycosis than skin infection. In cases of recurrent post-treatment onychomycosis, strain fingerprinting could determine whether the original isolate is responsible for re-infection (i.e., whether treatment failed) or a new strain has been acquired. There are several potential epidemiological applications for typing, including identifying strains endemic to a particular area and determining common sources of infection. Attempts to differentiate isolates of *T. rubrum* using molecular methods such as PCR fingerprinting, arbitrarily primed PCR and random amplification of polymorphic DNA²⁻⁴ have been largely unsuccessful, and the extent of interstrain genomic variation within this apparently clonal species

appears limited². Jackson *et al.*⁵ identified substantial polymorphism in the ribosomal DNA (rDNA) repeat region of *T. rubrum*. They described two novel tandemly repetitive subelements (TRSs), TRS-1 and TRS-2, located in the *T. rubrum* rDNA nontranscribed spacer (NTS). Specific amplification of the NTS region identified strain-specific variations in the copy number of these different subrepeat elements. The characteristic fingerprints generated by this PCR assay provided a rapid, stable, and reproducible molecular typing system to study the epidemiology and pathogenicity of *T. rubrum* infection.

In this study, we used this molecular typing method to identify *T. rubrum* isolates from several different clinical specimens of Iranian patients.

Materials and Methods

Dermatophyte isolates

23 Isolates were recovered from a range of clinical lesions. All isolates were identified to species level using standard mycological procedures. The strains of *T. rubrum* and their origins are listed in Table 1.

Table 1. The site and TRS type of *T. rubrum* strains

No. of isolates	Site of isolation	TRS-1	TRS-2
6	Skin, groin	2	II
3	Skin, groin	3	II
1	Skin, groin	1	II
1	Nail	4	II
1	Skin, arm	3	II
1	Skin, arm	4	II
1	Toenail	2	II
1	Toenail	3	II
2	Toenail	1	II
1	Skin, knee	5	II
1	Skin, leg	1	I
1	Skin, leg	2	II
1	Skin, foot	1	II
1	Skin, foot	1	I
1	Skin, foot	3	II

Extraction of genomic DNA

DNA extraction was performed directly from primary plate cultures. Approximately 2 mg of hyphal tissue was placed in a 0.6 ml microcentrifuge tube containing 500 mg of 0.45 to 0.5-mm-diameter sterile glass beads (B. Braun Biotech International GmbH, Melsungen, Germany). Three

hundred microliters of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA, 150 mM NaCl, 1% sodium dodecyl sulfate, 50 µg of proteinase K per ml) was added, and two freeze-thaw cycles at -80°C were carried out. The sample was vortexed for 3 minutes and incubated for 1 hour at 60°C. The base of the microcentrifuge tube was pierced with a sterile needle and placed in a 1.5-ml microcentrifuge tube, and the liquid contents were transferred to the larger tube by centrifugation (13,000 ×g, 30 s). Fifty microliters of 5 M sodium perchlorate was added, and incubation continued for a further 15 minutes at 60°C. Nucleic acids were phenol-chloroform extracted, ethanol precipitated, and washed and the purified DNA was re-suspended in 30 µl of sterile water.

PCR assay

Two primer pairs were designed from sequences flanking the two repetitive elements, TRS-1 and TRS-2. Primers TrNTSF-2 (5'-ACCGTATTAAGCTAGCGCTGC-3') and TrNTSR-4 (5'-TGCCACTTCGATTAGGAGGC-3') were used to amplify TRS-1, and primers TrNTSR-1 (5'-CTCAGTCGAACCGTGAGGC-3') and TrNTSC-1 (5'-CGAGACCACGTGATACATGCG-3') to amplify TRS-2. For amplification of TRS-1, a reaction mixture was made containing reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 10 µl Q solution, 1.5 mM magnesium chloride, a 0.125 mM concentration of each dNTP (dATP, dCTP, dGTP, and dTTP), 100 pmol each of primers TrNTSF-2 and TrNTSR-4, 0.5 U of Taq polymerase (QIAGEN Ltd., UK), and 5 µl of diluted template DNA. The final reaction volume was made up to 50 µl with pure water. The amplification reaction was carried out on a thermal cycler (Techne) with an initial denaturation for 2 minutes at 94°C, followed by 30 cycles of primer annealing at 58°C for 0.5 minute, extension at 72°C for 3 minutes, and denaturation at 94°C for 0.5 minute. A terminal extension step of 72°C for 10 minutes completed the PCR. Amplification of TRS-2 was carried out using the same reaction mixture as TRS-1 but without BSA. Cycling conditions for this reaction included denaturation for 1 minute at 94°C, followed by 30 cycles of primer annealing at 55°C for 0.5 minute, extension at 72°C for 2 minutes, denaturation at 94°C for 0.5 minute, and a terminal extension step of 72°C for 10 minutes. Amplification products were separated by electrophoresis in 2% agarose gels, visualized by staining with ethidium bromide, and photographed.

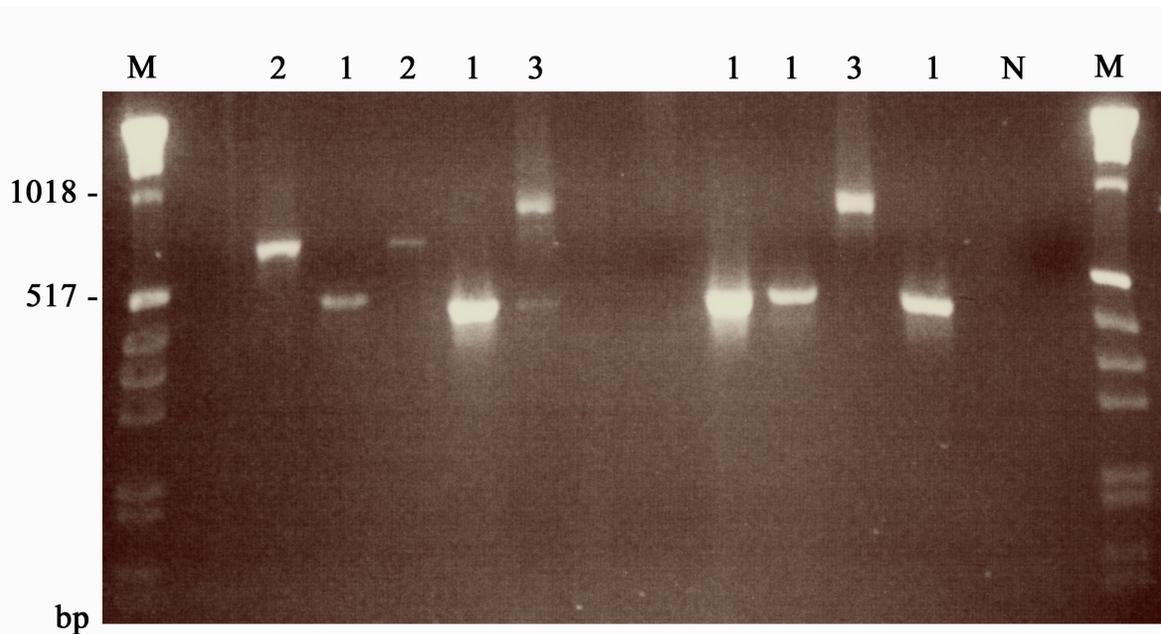


Figure 1. Amplification of the TRS-1 subrepeat element produced different PCR types from 23 clinical isolates of *T. rubrum*; M: molecular weight marker.

Results

On the basis of copy numbers of TRS-1 and TRS-2, 8 of our 23 samples were type 2 & II, respectively. Six of them were type 3 & II, four isolates were type 1 & II, two isolates were type 4 & II, two isolates were type 1 & I and one isolate was type 5 & II (figures. 1 & 2).

Five PCR type TRS-1 (1, 2, 3, 4 & 5) were present in 23 samples originating from the Iranian patients. The most common PCR type of TRS-1 (type 2) was present in 8 samples (34.8%). Six strains (26.1%) were PCR type 1, six strains (26.1%) were PCR type 3 and 1 strain (4.3%) was PCR type 5.

All of these isolates produced two band patterns characteristic of PCR types TRS-2 (I & II). Only two strains were type II.

Discussion

The identification of *T. rubrum* at the strain level might help considerably in the treatment and prophylaxis of dermatophytosis⁶. Molecular typing could be particularly useful in solving epidemiological questions⁷, for example revealing infection routes, common sources of infection and areas of dissemination, as well as determining whether the original isolate is responsible for re-infection or a new strain has been acquired^{8,9}.

Molecular biology techniques, such as PCR, RFLP, randomly amplified polymorphic DNA (RAPD)

analysis and Southern blotting, have led to dramatic progresses in distinguishing among species and strains⁷.

Studies by Gräser et al.¹⁰ have further highlighted the uniformity in the *T. rubrum* complex by comparing the morphological and physiological features with the results of sequencing of the internal transcribed spacer (ITS) region of the ribosomal operon, PCR fingerprinting and amplified fragment length polymorphism analysis.

T. rubrum has subrepeat elements in the rDNA and it has been demonstrated that variations in the copy number of these elements are useful for strain

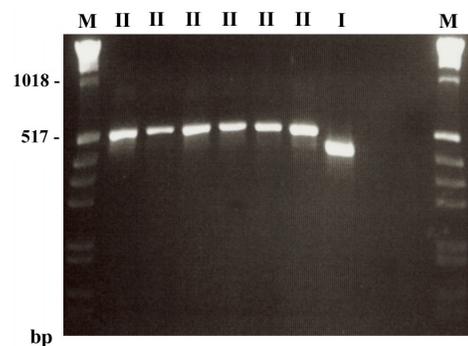


Figure 2. Amplification of the TRS-2 subrepeat element produced different PCR types from 23 clinical isolates of *T. rubrum*; M: molecular weight marker.

identification⁵. NTS region contains two sets of TRSs. Tandemly repetitive copies of a 200-bp element (*T. rubrum* TRS-1) were identified, with an adjacent 49-bp partial repeat. A second set of shorter subrepeat elements (*T. rubrum* TRS-2) was present as two tandem copies, 77 bp in length, followed by a third 46-bp partial repeat.

Jackson *et al.*⁵ described intraspecific variation in *T. rubrum* strains by the amplification of tandemly repetitive subelements (TRS-1) from the rDNA nontranscribed spacer (NTS) region, 21 TRS-1 PCR types being recognized in 101 clinical isolates. The copy number of TRS-2 was 0 to 3 repeats per cistron, with a majority of isolates having two copies of this element. Eleven isolates were polymorphic for TRS-2, and in combination, 23 separate PCR types were recognized by amplification of both TRS-1 and TRS-2.

In a study by Baeza *et al.*⁷, three TRS-1 PCR types (1, 2 and 3) were found and 60 strains (89.5%) were classified as PCR type 1, a higher proportion than 40 % reported for the most common type seen by Jackson *et al.*⁵, but in our study only 26.1% of isolates were found as PCR type 1, which is considerably lower than in their works. Amongst isolates in the United Kingdom and Brazil, the largest number of strains was TRS-1 type 1^{5,7}. One explanation for this pattern assumes that multiplication in TRS-1 copy number by unequal crossover is a rare event.

In this study, most of *T. rubrum* isolates were type 2 & II, dissimilar to European studies where type 1 & II has been the most common. The present study showed that 26.1% of Iranian isolates were TRS-1 PCR type 1 in contrast with a previous study that has demonstrated a much lower prevalence in Asians (5%)⁵.

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