

**Short Report**

**Identification of *Trichophyton rubrum* by Nested PCR Analysis from Paraffin Embedded Specimen in Trichophytia Profunda Acuta of the Glabrous Skin**

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**Abstract**

Trichophytia profunda acuta of the glabrous skin (TPAGS) arose in a 67-year-old Japanese man. The patient presented indurated erythematous plaques and nodules on his left forearm. Direct microscopic examination of the scale in KOH preparation was negative for fungal elements, and culture for dermatophytes was also negative. Although fungal infection could not be proven in hematoxylin-eosin stained sections, deep-cut sections of the biopsied skin lesion with PAS stain revealed the ectothrix presence of fungal elements. Nested PCR was done with *Trichophyton* specific primers directed to internal transcribed spacer gene 1 (ITS1), using template DNA obtained from formalin fixed, paraffin embedded skin sections. A single band corresponding to *T. rubrum* was obtained, and the etiological agent was thus identified. KOH tests and cultures may often turn out unsuccessful, perhaps reflecting the hair follicle dominant fungus growth in TPAGS. Although these tests are most important for diagnosis of TPAGS, nested PCR using paraffin embedded skin sections may be an alternative method to identify the etiological agent.

**Key words:** trichophytia profunda acuta of the glabrous skin, *Trichophyton rubrum*, inflammatory tinea corporis, PCR

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**Introduction**

Trichophytia profunda acuta of the glabrous skin (TPAGS) is an inflammatory variant of tinea corporis and receives special focus in Japan because of its deep follicular involvement and unusual clinical presentations<sup>1)</sup>. Skin biopsies are often done suspecting diseases other than dermatophytoses, and histopathological findings may come as a surprise for many clinicians, as the clinical feature may resemble a variety of skin diseases ranging from eczema to granulomatous diseases. Here, we report a case of TPAGS with unsuccessful fungus culture, in which the pathogenic agent was identified as *Trichophyton rubrum* by nested PCR using paraffin embedded skin sections.

**Case report**

A 67-year-old Japanese man visited our department presenting a pruritic eruption on the arm. His referring physician had treated him with short-term oral itraconazole and topical 2% ketoconazole (Nizoral cream®) with some effect, but the lesion was recurrent. Examination revealed an erythematous lesion on the left forearm, a part of which was annular with slightly elevated borders in the periphery, and dry scales on the surface (Fig. 1A). Direct observation of the scales under a light microscope in KOH preparation was negative for fungal elements. Differential diagnosis included granuloma annulare, sarcoidosis, chromomycosis, and TPAGS. At the time of skin biopsy, superficial inflammation had somewhat subsided, but the granulomatous papules were more prominent (Fig. 1B).

Histopathology in a hematoxylin-eosin stained

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Fig. 1. a and b, Indurated, slightly scaly annular erythema on the left forearm at the time of first visit. c and d, The superficial inflammation had subsided at the time of skin biopsy, leaving follicles oriented granulomatous papules.



Fig. 2. Deep-cut sections with PAS stain revealed numerous spores (arrow) in the hair follicle with ectothrix infection.

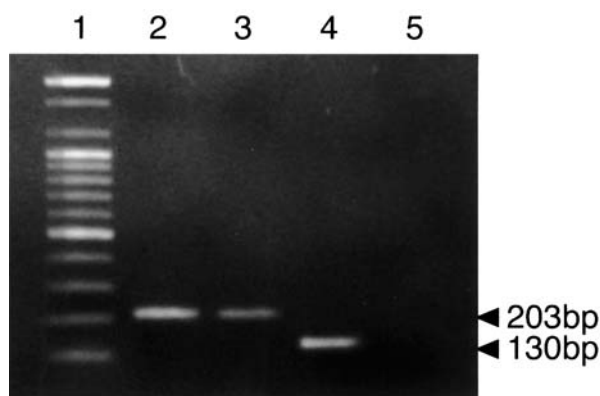


Fig. 3. Electrophoretic gel of the nested PCR products using *Trichophyton* species-specific primers. Lane 1, 100 bp ladder marker; lane 2, *T. rubrum* positive control; lane 3, sample; lane 4, *T. mentagrophytes* positive control; lane 5, sample. A 203-bp PCR product specific to *T. rubrum* was amplified.

section revealed hyperkeratosis, acanthosis, and a dense inflammatory infiltrate consisting of lymphocytes, neutrophils, histiocytes, and eosinophils. These infiltrates were mostly oriented around the hair follicles. Since the histopathological picture suggested, but showed no apparent evidence of fungal infection, deep-cut sections with PAS stain were prepared. Ectothrix presence of numerous round, PAS positive fungal elements were found in a hair follicle (Fig. 2). From these clinical and histopathological findings, the lesion was diagnosed as TPAGS. It was assumed to have infected from his pre-existing tinea pedis, although no culture was performed and the causative agent was not determined. The patient was given 125 mg/day of oral terbinafine and 1% terbinafine cream, and the lesion cleared up in two weeks. However, fungus culture that was done at the time of skin biopsy had turned out to be unsuccessful, and the etiological agent remained unknown.

#### Material and Methods

**Primer design:** Original primers targeting the internal transcribed spacer (ITS) region were designed according to the sequences available in Genbank, under the accession numbers Z97993 and AF170472 for *T. rubrum* and Z98000 and Z97995 for *T. mentagrophytes*. The primers for the first PCR were Univ-F (CTACCGATTGAATGGCTCAGTG), and Univ-R (GAACCAAGAGATCCGTTGTTG). This primer pair is able to detect *T. rubrum*, *T. mentagrophytes*, and *T. tonsurans*, according to the frequency of the causative fungi in Japan. Primers T.rub-F (GACCGACGTTCCATCAGGGGT) and T.rub-R (TCAGACTGACAGCTCTTCAGAG) for *T. rubrum*, or T.ment-F (CAAACGTCGGTC-AGGGTGAGC) and T.ment-R (TAGCCACTAAA-GAGAGGCTCGC) for *T. mentagrophytes* were designed for the second PCR. The expected lengths of the PCR products for the two primer sets were 203 and 130 bp, respectively. The specificities of the primers were confirmed by the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) via internet, courtesy of the National Center for Biotechnology Information (NCBI).

**Nested PCR:** A 3  $\mu$ m thick paraffin embedded skin section obtained by biopsy was used for the source of DNA extraction. After suspending the section in lysis buffer (100 mM Tris-HCl [pH 8.0], 30 mM EDTA [pH 8.0], and 0.5% sodium dodecyl sulfate)<sup>2)</sup> without a deparaffinization step, DNA was extracted by ethanol

precipitation and re-suspended in TE buffer. Extracted DNA (20  $\mu$ l) from sample was added to 30  $\mu$ l of the PCR master mixture, which consisted of 5  $\mu$ l of 10 X PCR buffer, 4  $\mu$ l of 200  $\mu$ M deoxynucleoside triphosphates, 30 pmol of each primer, and 2.5 U of Ex *Taq* DNA polymerase (Takara, Shiga). PCR was performed with an initial denaturation of 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 1 min at 56°C, and 30 s at 72°C, and a final extension at 72°C for 10 min with primers Univ-F and Univ-R. In the nested PCR step, 1  $\mu$ l of the first amplification product was added to a new reaction mixture with the same composition as the first. PCR consisted of an initial denaturation of 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and a final extension at 72°C for 10 min with primers T.rub-F and T.rub-R for *T. rubrum* or T.ment-F and T.ment-R for *T. mentagrophytes*. PCR product was separated in 1.5% agarose gel, stained with ethidium bromide and visualized under a UV illuminator.

**Result:** A 203 bp band corresponding to *T. rubrum* was observed not in the first, but in the second PCR (Fig. 3). Second PCR for *T. mentagrophytes* was negative, and since the above results were obtained, PCR analysis for *T. tonsurans* was not done. Since nested PCR increases sensitivity but may sometimes result in non-specific amplifications, the PCR product was cloned using a TA Cloning Kit (Invitrogen Corp., Carlsbad, Calif.), and sequenced with an ABI PRISM Cycle Sequencing Kit (Applied Biosystems, Foster, Calif.) according to the manufacturers' instructions. DNA sequence of the obtained clone completely matched that of *T. rubrum* (AF170472), and the etiological agent in the presented case of TPAGS was thus identified.

#### Discussion

While the entity TPAGS has not been globally accepted, it is distinguished from inflammatory tinea corporis in Japan. TPAGS often manifests as indurated erythematous plaque with slight scales and nodules arising on the limbs in the majority of cases<sup>3)</sup>. It may thus be distinguished from kerion of the glabrous skin, when kerion is defined as "intense, boggy, violaceous reaction often with suppuration"<sup>4, 5)</sup>. Iatrogenic mechanisms cannot be denied in some cases of TPAGS, because patients often have a history of treatment with topical steroid prior to diagnosis, and discontinuation of

topical steroids often leads to spontaneous remission<sup>3)</sup>.

KOH tests and cultures may tend to yield negative results from this clinical type, perhaps reflecting the follicular dominant growth of fungi. It would be strongly preferable to identify the etiological agent not only for accurate diagnosis, but also for post-therapeutic strategies in prevention, especially in those with *Microsporum canis* or *T. tonsurans* infections, the latter currently showing a massive outbreak in Japan<sup>6, 7)</sup>. Identification by conventional methods may, however, be difficult in situations such as ours, where anti-fungal therapy is started empirically, and the culture done on the initial visit turns out to be unsuccessful.

Meanwhile, molecular biology has enabled giant steps in our understanding of taxonomy by the application of genetic methods to identify microbial agents, including human pathogenic fungi<sup>8, 9)</sup>. The database on rRNA gene is growing rapidly. Sequence data is obtainable via internet to design specific primers or easily compare the sequence of interest to the vast number of registered sequences in Genbank. Given this fact and the improving techniques for DNA isolation or PCR, it is now possible to retrospectively determine the causative agent from stored clinical samples such as sera or paraffin embedded sections. Although fungus culture yielded a negative result, we were able to identify the etiological fungus as *T. rubrum* from paraffin embedded sections.

Results of positive PCR must be carefully interpreted especially when clinical samples are used as templates, because conventional PCR does not distinguish active infection from colonization. Isolation of fungi by culture remains to be the strongest evidence in diagnosis and identification of specific pathogens. PCR analysis, however, may be an alternative method to

determine identification of culture negative, but biopsy confirmed pathological specimens.

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