Molecular Epidemiology of *Trichophyton tonsurans* Isolated in Japan Using RFLP Analysis of Non-Transcribed Spacer Regions of Ribosomal RNA Genes

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SUMMARY: *Trichophyton tonsurans* has been reported to be the causative agent of an epidemic of tinea corporis and tinea capitis among Japanese judoists and wrestlers. A molecular method using restriction enzyme analysis of PCR-amplified fragments targeting the non-transcribed spacer (NTS) region of ribosomal RNA genes in fungal nuclei was applied to a total of 232 strains of *T. tonsurans* isolated in Japan. Six molecular types, i.e., NTS types I, II, III, IV, V, and VI, were clearly detected in restriction analysis of fragments digested with *Mva*I and *Ava*I together. Of the 232 strains, 199 were classified as NTS I, 21 as NTS II, 7 as NTS III, 3 as NTS IV, 1 as type V, and 1 as type VI. Whereas the NTS I strains were found nationwide, most of the NTS II and NTS III strains were limited to central Japan. Of 164 strains isolated from judoists, 160 were classified as NTS I, which suggests that the majority of the cases were caused by a clonal lineage. On the other hand, the 48 strains isolated from wrestlers showed more variety, with 27 strains classified as NTS I, 17 as NTS II, and 4 as NTS III. We concluded that the epidemic was caused by at least three lineages of *T. tonsurans*. NTS VI strains, the major molecular type among sporadically isolated strains, were not observed among the epidemic strains, and strains of this type did not contribute to the present epidemic.

INTRODUCTION

*Trichophyton tonsurans* is a ubiquitous dermatophyte species that causes tinea capitis and tinea corporis in Europe and America, especially among schoolchildren, students, and athletes who come into direct bodily contact with others. *T. tonsurans* had been reported to be responsible for only several sporadic cases of tinea capitis in Japan, but recently it has been identified as the causative agent of an epidemic of tinea corporis and tinea capitis among wrestlers and judoists in Japan (1,2).

Species-level identification of *T. tonsurans* is an important part of laboratory investigations carried out to control such epidemics. Identifying the species by conventional morphological methods is usually simple, and the use of molecular methods, such as random amplified polymorphic DNA analysis (3) or PCR restriction fragment length polymorphism (PCR-RFLP) analysis targeting the internal transcribed spacer (ITS) region (4), is helpful in confirming the identification.

However, there are occasions when subspecies-level differentiation is required to elucidate the transmission of *T. tonsurans* strains between people. Thus, sensitive techniques are needed for strain identification or the typing of isolates of *T. tonsurans*. Analysis of the non-transcribed spacer (NTS) region of the ribosomal RNA (rRNA) genes in fungal nuclei has been reported to be a reliable technique for strain typing *Trichophyton rubrum* (5,6), *Arthroderma benhamiae* (7), and *Trichophyton mentagrophytes* (8,9), and PCR primers for amplifying NTS regions of *T. tonsurans* have recently been reported by several authors (10-12). In the present study, we applied a primer set for PCR-RFLP analysis for subtyping *T. tonsurans* strains isolated in Japan, and attempted to understand the background of the epidemic.

MATERIALS AND METHODS

Fungal strains (Table 1): We examined *T. tonsurans* isolates obtained between 2001 and 2006 from 232 people at 58 institutes or clinics in 27 prefectures in 7 regions, covering most of Japan. The regions and the number of cases in each were: Hokkaido, 4; Tohoku (northern part of Honshu island: Aomori, Akita, Yamagata, Iwate, Miyagi), 63; Kanto-Koshinetsu (central-east Honshu island: Tokyo, Kanagawa, Niigata, Ibaraki, Yamanashi), 29; Chubu (central Honshu island: Aichi, Shizuoka, Gifu, Mie, Toyama, Ishikawa, Fukui), 47; Kansai (central-western Honshu island: Shiga, Kyoto, Osaka, Hyogo, Nara), 71; Chugoku (western Honshu island: Okayama, Hiroshima), 2; and Kyushu (Kyushu island: Saga, Nagasaki), 16. Most of the isolates were cultured from lesions obtained from the scalp using a hairbrush sampling method (2). All but 7 of the 232 people either engaged in contact sports or were family members or intimate friends of contact sports players. The 7 other strains were isolated from cases that arose sporadically. The sports and the number of cases associated with each were: judo, 164; wrestling, 48; junior sumo, 2; Brazilian wrestling, 1; unknown (not documented), 10.

To identify the strains, we first observed the gross features of their colony morphology, and then confirmed that the
strains were compatible with \emph{T. tonsurans} by PCR-RFLP analysis of the ITS regions of rRNA genes (Figure 1). The methods used for the analysis of ITS are described below.

**Preparation of template DNA, PCR conditions, and RFLP analysis:** Total cellular DNA was extracted from colonies cultured on Sabouraud's dextrose agar slants or plates with or without antibiotics by a rapid preparation method (13). Briefly, a single colony or a small amount of a mycelial mat (wet weight, 10 to 50 mg) was added to 300 μl of lysis buffer (200 mM Tris-HCl, pH 8, 0.5% sodium dodecyl sulfate, 250 mM NaCl, 25 mM EDTA) and ground with a conical grinder for 10 to 30 s. After the addition of 150 μl of 3.0 M sodium acetate, the homogenate was centrifuged at 10,000 × g for 10 min. The supernatant was successively extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform, and the DNA was precipitated by adding the same volume of isopropanol. The precipitate was washed with 70% ethanol, dried, and dissolved in 30 μl of distilled water, and aliquots of 1 μl of the resulting solution were used as the template in subsequent PCR.

To confirm the strains as \emph{T. tonsurans}, universal primer pair targeting of ITS regions, i.e., ITS1 (5'-TCCGTAGGGTGAACC TGCGG) and ITS4 (5'-TCTTCCGCTTAATGATATGC) (14), was used (Figure 1). The thermal cycler was programmed for 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C. The amplicons were digested with \emph{MvaI} and \emph{AvrII} (Toyobo), then electrophoresed on 5% polyacrylamide gels. First, we selected the restriction enzyme \emph{MvaI}, which clearly showed 6 molecular types, namely \emph{AvrII}1, 2, 3, 4, 5, and 6. However, \emph{AvrII}1, 3, 4, and 6 had no restriction site in the ITS fragments, and \emph{AvrII}1 failed to digest the 726-bp and 708-bp fragments. Therefore, \emph{MvaI} was also used. It showed 5 molecular types, \emph{MvaI}:1, 2, 3, 4, and 5, which showed 2 to 5 fragments on the gels (Figure 2). Close relationships were observed between the types determined with these two enzymes: i.e., between \emph{MvaI}:1 and \emph{AvrII}:1 or 3; \emph{MvaI}:2 and \emph{AvrII}:2; \emph{MvaI}:3 and \emph{AvrII}:4; \emph{MvaI}:4 and \emph{AvrII}:5; and \emph{MvaI}:5 and \emph{AvrII}:6. Digestion with \emph{MvaI} and \emph{AvrII} together showed 6 molecular types, NTS I, II, III, IV, V, and VI (Table 1).

![Fig. 1. Structure of ribosomal RNA genes and targeted regions in the study.](image)

**RESULTS**

It took less than 8 h to obtain the banding profiles of ITS from fungal mycelia: 2 h for the extraction and preparation of the template DNAs, 3 h for PCR, 1 h for digestion, 1 h for electrophoresis, and 30 min for the staining and observation of the bands.

The amplified fragments derived from the ITS regions were between 183 bp and 726 bp in length. Restriction enzyme analysis was performed to evaluate the sizes of two of the fragments, which were 726 bp and 708 bp in length, because they could not be accurately and reliably evaluated on 5% polyacrylamide gels. First, we selected the restriction enzyme \emph{MvaI}, which clearly showed 6 molecular types, namely \emph{AvrII}1, 2, 3, 4, 5, and 6. However, \emph{AvrII}1, 3, 4, and 6 had no restriction site in the NTS fragments, and \emph{AvrII}1 failed to digest the 726-bp and 708-bp fragments. Therefore, \emph{MvaI} was also used. It showed 5 molecular types, \emph{MvaI}:1, 2, 3, 4, and 5, which showed 2 to 5 fragments on the gels (Figure 2). Close relationships were observed between the types determined with these two enzymes: i.e., between \emph{MvaI}:1 and \emph{AvrII}:1 or 3; \emph{MvaI}:2 and \emph{AvrII}:2; \emph{MvaI}:3 and \emph{AvrII}:4; \emph{MvaI}:4 and \emph{AvrII}:5; and \emph{MvaI}:5 and \emph{AvrII}:6. Digestion with \emph{MvaI} and \emph{AvrII} together showed 6 molecular types, NTS I, II, III, IV, V, and VI (Table 1).

Of all 232 strains, 199 were NTS I, 21 were NTS II, 7 were NTS III, 3 were NTS IV, 1 was NTS V, and 1 was NTS VI. Of the 164 strains isolated from the judoists, 98% (160/164) were NTS I, suggesting that most cases were caused by a clonal lineage. In contrast, the 48 strains isolated from the wrestlers were diverse, with NTS I accounting for only 56% (27/48) of them, and NTS II and III accounting for 35% (17/
found in Tohoku. One strain classified as NTS V was isolated from a Japanese boy with kerion celsi, and one strain classified as NTS IV was isolated from a girl with tinea capitis, who was an immigrant from Peru.

Nucleotide sequence analysis of the NTS regions of the representative strains of the 6 molecular types of *T. tonsurans* were used to estimate restriction enzyme profiles for *Mva*I and *Ava*I. NTS I (represented by KMU 4417) was 425 bp in length, including the sites recognized by the primers (DDBJ accession no. AB275311). Regarding the PCR amplicons, NTS II (represented by KMU 4250) was 480 bp in length (DDBJ accession no. AB275312), NTS III (represented by KMU 4720) was 670 bp (DDBJ accession no. AB275313) and a band about 420 bp in length, NTS IV (represented by KMU 4855) was 726 bp in length (DDBJ accession no. AB275315), and NTS VI (KMU 4253) was 708 bp in length (DDBJ accession no. AB275316). The banding profiles (Table 2), estimated from the sequence data were compatible with the results of electrophoresis (Figure 2). Sequence analysis also confirmed the lack of *Ava*I sites in types NTS I, III, IV, and VI.

**DISCUSSION**

*T. tonsurans* is a very genetically homogenous species. Even the highly sensitive random amplified polymorphic DNA method failed to find intraspecies polymorphisms (3). Sequence analysis of ITS regions of rRNA genes showed only two molecular types based on a small number of nucleotide polymorphisms in 7 Japanese isolates, and the regions are now used as good markers for species-level identification (4). Recently, several studies using NTS of rRNA genes successfully detected molecular types among *T. tonsurans* isolates (10-12). Gaedigk et al. (10) detected 5 variants differing in size by PCR targeting the variable internal repeat (VIR) region in NTS and 7 sub-variants derived from single nucleotide polymorphisms detected by RFLP analyses using 7 restriction enzymes. A total of 12 genotypes could be discriminated among 92 strains acquired from 6 US microbiology laboratories. Abliz et al. (11) studied the almost full-length NTS, approximately 2.4 to 2.9 kb in length, in a PCR-RFLP study and found 4 genotypes among 19 strains isolated from Brazil, Italy, and China. Sugita et al. (12) used the same primer pair reported by Gaedigk et al. (10) and detected 2 variants in length in addition to the 5 variants reported by Gaedigk et al. (10) using sequence analysis. The primer pair used in the

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**Table 2. Molecular typing definition in the present study and correlations with previous reports**

<table>
<thead>
<tr>
<th>Present Study (Size of PCR product/digested fragments, estimated)</th>
<th>Gaedigk et al. (10)</th>
<th>Sugita et al. (12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTS I: <em>Mva</em>I: 1/<em>Ava</em>I: 1</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>NTS II: <em>Mva</em>I: 2/<em>Ava</em>I: 2</td>
<td>II</td>
<td>IV</td>
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<tr>
<td>NTS III: <em>Mva</em>I: 1/<em>Ava</em>I: 3</td>
<td>III</td>
<td>V</td>
</tr>
<tr>
<td>NTS IV: <em>Mva</em>I: 3/<em>Ava</em>I: 4</td>
<td>none</td>
<td>I</td>
</tr>
<tr>
<td>NTS V: <em>Mva</em>I: 4/<em>Ava</em>I: 5</td>
<td>IV</td>
<td>VI</td>
</tr>
<tr>
<td>NTS VI: <em>Mva</em>I: 5/<em>Ava</em>I: 6</td>
<td>none</td>
<td>none</td>
</tr>
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*: doublet.

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**Fig. 2. Electrophoresis of PCR-RFLP products. Lanes for *Mva*I 1, KMU 4948; 2, KMU 4250; 3, KMU 3313; 4, KMU 4855; 5, KMU 4253. Lanes for *Ava*I 1, KMU 4417; 2, KMU 4250; 3, KMU 4720; 4, KMU 3313; 5, KMU 4855; 6, KMU 4253.**

**Fig. 3. Geographical distribution of NTS II strains.**

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48) and 8% (4/48), respectively. Both isolates from the junior sumo wrestlers were NTS I. The 7 sporadically isolated strains from people not associated with contact sports were classified as either NTS II, NTS IV, NTS V or NTS VI.

The NTS I strains were found in all 7 regions, while NTS II was found in two neighboring regions and NTS III mainly in just one region, with 16 of the 21 NTS II strains found in Chubu or Kansai (Figure 3), and 5 of the 7 NTS III strains
isolated from an epidemic on a high school wrestling team may have been infected with NTS I strains. In fact, strains that many high school wrestling teams accepted judoists, who nation of the polymorphisms in wrestling isolates could be studied to confirm this geographic distinction. One expla-

accounting for little over half and NTS II for just over a third of the cases. In addition, NTS I seemed to be widely distributed, but the NTS II strains were mainly restricted to Miyagi, and NTS III was isolated from Okayama, and NTS IV was from Tokyo and Miyagi. Sugita et al. (12) examined 101 isolates from Japanese judoists and demonstrated the homogeneity of the genotype, which corresponded to NTS I in the present study. The molecular polymorphisms in this study were found when the areas from which the isolates were obtained were expanded and the number of strains was increased.

The epidemic among judoists in Japan started around 1999, and spread explosively nationwide around 2001 (15). The epidemic is thought to have started among high school teams, and then spread to middle and junior high school teams. The untreated high school players are thought to have spread it among both university teams and the general public. Bodily contact during training and competitions is thought to be a major factor in the spread of the disease. Three molecular types were detected among the isolates from the judoists--NTS I, II, and III. The majority of the strains (160 of 164 strains) were NTS I. NTS II was isolated from Yamagata and Okayama, and NTS III was isolated from Tokyo and Miyagi. Sugita et al. (12) examined 101 isolates from Japanese judoists and demonstrated the homogeneity of the genotype, which corresponded to NTS I in the present study. The molecular polymorphisms in this study were found when the areas from which the isolates were obtained were expanded and the number of strains was increased.

The epidemic among wrestlers has a rather complicated history. The earliest observation of the epidemic was recorded in 2001 (1,15); however, an epidemic on a high school wrestling team was noticed by a dermatologist in Toyama, Japan, between 1994 and 1995 (15). In the present study, judging from the molecular types of the isolates, the history of the wrestlers’ epidemic may be somewhat more complex than that of the judoists’ epidemic. Though types NTS I, II, and III were found in both groups, in the case of the judoists’, nearly all the strains were of just one type, NTS I, whereas in the case of the wrestlers, there was more variation, with NTS I accounting for little over half and NTS II for just over a third of the cases. In addition, NTS I seemed to be widely distributed, but the NTS II strains were mainly restricted to Chubu or Kansai (Figure 3), although more strains need to be studied to confirm this geographic distinction. One explanation of the polymorphisms in wrestling isolates could be that many high school wrestling teams accepted judoists, who may have been infected with NTS I strains. In fact, strains isolated from an epidemic on a high school wrestling team (1) were later shown to be composed of two molecular types, NTS I and II, at the same time. Indeed, one patient with tinea capitis was on that team had been a member of a junior high school judo team. Transmission by infected players participating in contact sports was the main cause of the spread of the epidemic, and there is concern that the epidemic of NTS I may occur among sumo wrestlers, who sometimes also train with judoists (16).

NTS IV has been isolated from sporadic cases in Japan, and the same banding profile was observed in a registered T. tonsurans strain, CBS129.35 (data not shown), isolated in Japan in 1935 (12). NTS IV T. tonsurans is considered to be a domestic flora, and no NTS IV strains were isolated from the epidemic cases. Possible routes of transmission of domestic and imported T. tonsurans are shown in Figure 4.

The origin of the epidemic strains in Japan is of epidemiological interest. Sugita et al. postulated that T. tonsurans type II (NTS I in our typing system) had been brought into Japan from another country (12). As we have shown, no NTS IV strains were found among epidemic isolates, and no NTS I strains were found among the sporadic isolates. The results of the present study support the suggestion that the epidemic among judoists and wrestlers originated from outside Japan (2,12). In support of this suggestion, the presence of NTS I and II strains has been reported by Gaedigk et al., who classified 26 of 92 strains isolated in the US as Type I (NTS I in our typing system) and 48 of 92 strains as Type II (NTS II in our typing system) (10).
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