Original Article

Molecular Epidemiological Analysis of the Spreading Conditions of Trichophyton in Long-Term Care Facilities in Japan

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SUMMARY: Tinea pedis and tinea unguium are common infectious diseases, and many elderly people are reported to contract these infections. In this study, to investigate whether strains of the same origin are spreading inside a long-term care facility, we analyzed Trichophyton rubrum and Trichophyton mentagrophytes, isolated from the residents and staff at the facilities located in the Kanto area, using a genomic analytical method targeting tandem repeat regions in the nontranscribed spacer (NTS) region of ribosomal DNA. Five NTS types were confirmed in T. rubrum. T. rubrum of various types (types 1 to 5) was detected at each facility, but there was no isolate specific to one facility only. Eight NTS types of T. mentagrophytes were detected, and T. mentagrophytes that carried an NTS type that was confirmed at one facility only (types C4II, F4II, and D4II) was isolated. These T. mentagrophytes sequence types were isolated from several subjects residing at the same facility. This study proved that a T. mentagrophytes strain of the same type had spread in long-term care facilities. We believe in the importance of cleaning at a long-term care facility as a countermeasure to the spread of Trichophyton species.

INTRODUCTION

Trichophytosis is one of the major infectious diseases that invades human skin by the activation of an exotoxin (keratinase), and the target skin area is mainly located around the toes, where the causative fungus lodges stably. Trichophytosis of the foot is called tinea pedis. The causative fungi of tinea pedis are dermatophytes such as Trichophyton, Epidermophyton, and Microsporum. Especially, Trichophyton rubrum and Trichophyton mentagrophytes var. interdigitale are frequently isolated as pathogenic fungi (1).

Numerous epidemiological studies of skin infections have been conducted: The most prevalent disease in the past 3 surveys in Japan was tinea pedis, followed by tinea unguium (2–4). In addition, age-specific prevalence rates of tinea pedis and tinea unguium are constantly being investigated, and they are high in elderly people. An epidemiological study on Trichophyton collected from elderly people at long-term care facilities in 2016 revealed that approximately half of the elderly people shed Trichophyton from their feet, and we believe there is a risk for rapid spread of Trichophyton (5). Besides, it has been reported that elderly people with tinea pedis have experienced falling down significantly more frequently than the noninfected people have (6). Infec-

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Fungal isolates: In 2016, 23 isolates of T. rubrum (Table 1) and 20 isolates of T. mentagrophytes (Table 2) were collected from the feet of elderly subjects, who were aged 65 or more and the staff working at long-term care facilities (facilities A, B, C, D, and E) located in the
Table 1. Details of *T. rubrum* isolates used in this study

| Isolate no. | Facility | Collected | ITS profile | PCR type | NTS type | 1) |
|------------|----------|-----------|-------------|----------|----------|---|
| BGUTR2     | A        | the elderly | TR           | 1        | 1        | 1 |
| BGUTR3     | A        | the elderly | TR           | 1        | 1        | 1 |
| BGUTR4     | A        | the elderly | TR           | 1        | 1        | 1 |
| BGUTR16    | A        | the elderly | TR           | 2        | 1        | 2 |
| BGUTR5     | B        | the elderly | TR           | 3        | 1        | 3 |
| BGUTR6     | C        | the elderly | TR           | 2        | 1        | 2 |
| BGUTR9     | C        | the elderly | TR           | 3        | 1        | 3 |
| BGUTR10    | C        | the elderly | TR           | 3        | 1        | 3 |
| BGUTR8     | C        | the elderly | TR           | 4        | 1        | 4 |
| BGUTR7     | C        | the elderly | TR           | 5        | 1        | 5 |
| BGUTR14    | D        | the elderly | TR           | 1        | 1        | 1 |
| BGUTR15    | D        | the elderly | TR           | 2        | 1        | 2 |
| BGUTR11    | D        | the elderly | TR           | 3        | 1        | 3 |
| BGUTR12    | D        | the elderly | TR           | 4        | 1        | 4 |
| BGUTR13    | D        | the elderly | TR           | 4        | 1        | 4 |
| BGUTR18    | E        | the elderly | TR           | 1        | 1        | 1 |
| BGUTR19    | E        | the elderly | TR           | 1        | 1        | 1 |
| BGUTR24    | E        | the elderly | TR           | 2        | 1        | 2 |
| BGUTR21    | E        | the elderly | TR           | 3        | 1        | 3 |
| BGUTR17    | E        | the elderly | TR           | 4        | 1        | 4 |
| BGUTR20    | E        | the elderly | TR           | 4        | 1        | 4 |
| BGUTR23    | E        | the elderly | TR           | 5        | 1        | 5 |
| BGUTR22    | E        | the elderly | TR           | ND       | ND       | ND |

1) The combinations of TRS-1 and TRS-2 fingerprint patterns classified the isolates into five types. TR, *Trichophyton rubrum*; ND, not determined.

Table 2. Details of *T. mentagrophytes* isolates used in this study

| Isolate no. | Facility | Collected | ITS profile | PCR type | NTS type | 1) |
|------------|----------|-----------|-------------|----------|----------|---|
| BGUTM6     | A        | the elderly | TM          | D        | 1        | D11 |
| BGUTM21    | A        | the elderly | TM          | D        | 6        | D61 |
| BGUTM7     | B        | the elderly | TM          | D        | 2        | D21 |
| BGUTM8     | B        | the elderly | TM          | D        | 2        | D21 |
| BGUTM9     | B        | the elderly | TM          | D        | 2        | D21 |
| BGUTM10    | B        | the elderly | TM          | Z (new)  | 2        | Z21 |
| BGUTM11    | C        | the elderly | TM          | D        | 4        | D41 |
| BGUTM14    | C        | the elderly | TM          | D        | 4        | D41 |
| BGUTM12    | C        | the elderly | TM          | F        | 4        | F41 |
| BGUTM13    | C        | the elderly | TM          | F        | 4        | F41 |
| BGUTM16    | D        | the elderly | TM          | C        | 4        | C41 |
| BGUTM18    | D        | the elderly | TM          | C        | 4        | C41 |
| BGUTM19    | D        | staff      | TM          | C        | 4        | C41 |
| BGUTM17    | D        | the elderly | TM          | D        | 2        | D21 |
| BGUTM20    | D        | staff      | TM          | D        | 2        | D21 |
| BGUTM24    | E        | the elderly | TM          | C        | 8        | C81 |
| BGUTM22    | E        | the elderly | TM          | D        | 2        | D21 |
| BGUTM23    | E        | the elderly | TM          | D        | 2        | D21 |
| BGUTM25    | E        | the elderly | TM          | D        | 2        | D21 |
| BGUTM26    | E        | the elderly | TM          | D        | 2        | D21 |

1) The combinations of TmiS0, TmiS1 and TmiS2 fingerprint patterns classified the isolates into eight types. TM, *Trichophyton mentagrophytes*.

Kanto area, Japan. Sample collection and analysis in the study were approved by the ethics review committee of Bunkyo Gakuin University (Approval number: 2016-0010). These collected isolates were identified by the macroscopic examination of their colony morphology (pigmentation, the growth rate, and texture) on Sabouraud dextrose agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), microscopic examination of conidia, and PCR restriction fragment length polymorphism analysis of the amplified ITS region fragments by means of the MvaI restriction enzyme (8). The identified isolates were cryopreserved at −80°C until experiments were performed.

**DNA extraction method:** Conserved *Trichophyton* was applied to Sabouraud dextrose agar and cultured until colonies were confirmed. DNA extraction was performed with the modified Rapid Mini-Preparation method (9). A small amount of a fungus was added to the lysis solution (400 mM Tris-HCl Buffer [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% of sodium dodecyl sulfate) and heated at 100°C for 10 min. Potassium acetate was added to the fungus solution, and the mixture was incubated at −20°C for 5 min. After centrifugation (10,000 × g, 1 min), the supernatant was recovered. An equal volume of isopropyl alcohol was added to the supernatant and mixed, the mixture was centrifuged at 10,000 × g for 2 min, and the supernatant was removed. The precipitated DNA pellet was washed with 70% ethanol, centrifuged at 10,000 × g for 1 min, and the supernatant was again removed. The DNA pellet was air dried and dissolved in Tris-EDTA (TE) buffer.

**Analysis of molecular polymorphism:** The NTS region of ribosomal DNA was amplified by PCR, and molecular polymorphism was analyzed via electrophoretic patterns. Because the primers used were different among the fungal species, primers that amplify tandemly repetitive subelement 1 (TRS-1) and tandemly repetitive subelement 2 (TRS-2) in the NTS region were used with *T. rubrum* (Table 3) (10). Primers targeting the S0 tandem repeat region (TmiS0), the S1 tandem repeat region (TmiS1), and the S2 tandem repeat region (TmiS2) in the NTS region were used for *T. mentagrophytes*.

Table 3. PCR primers used for amplification of NTS regions

| Primer  | Sequence |
|---------|----------|
| *T. rubrum* | |
| tandemly repetitive subelement 1 (TRS-1) | |
| Forward primer TmNTSF-2 | 5-ACCGTATTAAAGCTAGCGCTGC-3 |
| Reverse primer TmNTSR-4 | 5-TGGCACTTGATAGGAGAGGC-3 |
| tandemly repetitive subelement 2 (TRS-2) | |
| Forward primer TmNTSR-1 | 5-CTCAGTCGAAACCCTGGCCG-3 |
| Reverse primer TmNTS-5 | 5-CGAGAGACACCTTGATACATGGCG-3 |
| *T. mentagrophytes* | |
| S0 tandem repeat region (TmiS0) | |
| Forward primer TmiS0F | 5-CGAGAGATACTCTGAGAAGATG-3 |
| Reverse primer TmiS0R | 5-GCAACGATACTGTGACACTCG-3 |
| S1 tandem repeat region (TmiS1) | |
| Forward primer TmiS1F | 5-CAGCACTTGACCTCTCAGTGC-3 |
| Reverse primer TmiS1R | 5-TCGCTGCCCTCGAAGAGCCAC-3 |
| S2 tandem repeat region (TmiS2) | |
| Forward primer TmiS2F | 5-GACCTCTATTCTAGTAGTGC-3 |
| Reverse primer TmiS2R | 5-CCATTCTACAGGAACTTTAG-3 |
PCR amplification conditions of TRS-1 were set to 30 cycles, consisting of 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min. The PCR amplification conditions for TRS-2 were set to 30 cycles, consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. In addition, the PCR amplification conditions for TmiS0, TmiS1, and TmiS2 were set to 30 cycles each, consisting of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s each. Each amplification product was applied to a 2% agarose gel, and electrophoresis was carried out; bands were visualized by staining with ethidium bromide. Polymorphisms of PCR products from each locus were identified by comparative gel electrophoresis, and the NTS type was determined (10, 11).

RESULTS

Molecular polymorphism analysis of T. rubrum: By amplifying TRS-1 and TRS-2 obtained from the NTS region of T. rubrum and by analyzing each electrophoretic pattern, five types (1, 2, 3, 4, and 5) based on TRS-1 were confirmed (Fig. 1). The type of TRS-1 for one isolate of BGUTR22 could not be determined. In terms of TRS-2, only one type (type I) was observed (Fig. 1).

Analysis was performed by combining the results of TRS-1 and TRS-2, and a total of five NTS types (1, 2, 3, 4, and 5) were identified. Table 1 shows the analysis results on the NTS type from 23 isolates of T. rubrum. BGUTR2, BGUTR3, and BGUTR4 isolated from subjects at facility A were type 1 of the same NTS type. At other facilities, partial concordance of the NTS type was confirmed. Nonetheless, T. rubrum of various types (types 1 to 5) was detected at each facility, and there were no isolates specific to one facility only. The isolation frequency of the NTS types of T. rubrum is shown in Table 4. The most prevalent confirmed NTS type was type 1 (six isolates), and type 5 was the least prevalent (two isolates).

Molecular polymorphism analysis of T. mentagrophytes: The NTS region of T. mentagrophytes was analyzed, and four PCR types (C, D, F, Z) were identified on the basis of TmiS0 (Fig. 2A). We confirmed five types (1, 2, 4, 6, and 8) on the basis of TmiS1 (Fig. 2A) and two types (I and II) based on TmiS2 (Fig. 2B). By analyzing the above PCR types, we identified a total of eight NTS types (C4II, C8II, D1II, D2II, D4II, D6I, F4II, and Z2II). The NTS types of T. mentagrophytes (20 isolates) collected from subjects residing at each facility are shown in Table 2. An isolate that had an NTS type confirmed at one facility only was detected at some

| Table 4. Frequency of NTS types of T. rubrum (A) and T. mentagrophytes (B) isolates |
|----------------------|----------------------|----------------------|
|                      | Number              | NTS type             | Number of isolates |
| A                    |                      |                      |                    |
| 1                    | 1                    | 6                     | (26.1%)            |
| 2                    | 3                    | 5                     | (21.7%)            |
| 3                    | 4                    | 5                     | (21.7%)            |
| 4                    | 2                    | 4                     | (17.4%)            |
| 5                    | 5                    | 2                     | (8.7%)             |
| 6                    | ND                   | 1                     | (4.3%)             |
| Total                |                      | 23                    |                    |
| B                    |                      |                      |                    |
| 1                    | D2II                 | 9                     | (45.0%)            |
| 2                    | C4II                 | 3                     | (15.0%)            |
| 3                    | D4II                 | 2                     | (10.0%)            |
| 4                    | F4II                 | 2                     | (10.0%)            |
| 5                    | C8II                 | 1                     | (5.0%)             |
| 6                    | D1II                 | 1                     | (5.0%)             |
| 7                    | D6I                  | 1                     | (5.0%)             |
| 8                    | Z2II                 | 1                     | (5.0%)             |
| Total                |                      | 20                    |                    |

ND, not determined.

Fig. 1. Agarose gel electrophoresis of PCR-amplified NTS regions (TRS-1 and TRS-2) from T. rubrum. Amplification of the TRS-1 element produced NTS types from 23 isolates of T. rubrum. Lanes: 1, M, molecular weight marker; 2, TRS-1 type 1; 3, TRS-1 type 2; 4, TRS-1 type 3; 5, TRS-1 type 4; 6, TRS-1 type 5; 7, M, molecular weight marker; 8, TRS-2 type I.

Fig. 2. Agarose gel electrophoresis of PCR-amplified NTS regions (TmiS0, TmiS1 and TmiS2) from T. mentagrophytes. PCR patterns determined NTS types from 20 isolates of T. mentagrophytes. (A) Lanes: 1, M, molecular weight marker; 2, TmiS0 type C; 3, TmiS0 type D; 4, TmiS0 type F; 5, TmiS0 type Z; 6, M, molecular weight marker; 7, TmiS1 type I; 8, TmiS1 type 2; 9, TmiS1 type 4; 10, TmiS1 type 6; 11, TmiS1 type 8. (B) Lanes: 1, M, molecular weight marker; 2, TmiS2 type I; 3, TmiS2 type II.
facilities (facility C, types D4II and F4II; facility D, type C4II), and these isolates were isolated from several subjects residing at the same facility. At facilities B and E, almost all the NTS types of *Trichophyton* were type D2II (3 out of 4 and 4 out of 5, respectively). The isolation frequencies of the NTS types are shown in Table 4. The most frequently isolated NTS type of *Trichophyton* was type D2II: this type accounted for approximately a half of the total.

**DISCUSSION**

Despite the large number of elderly people with tinea pedis and tinea unguium, there are few reports addressing conditions for the spread of *Trichophyton* at long-term care facilities in Japan. In addition, there is no study on *T. rubrum* or *T. mentagrophytes* of the same NTS type that spreads at a long-term care facility. In this study, by identifying TRS-1 and TRS-2 of *T. rubrum* and *T. mentagrophytes*, the number of isolates was approximately a half of the total (45%). The most prevalent NTS type of *T. mentagrophytes* was type D2II: this type accounted for approximately a half of the total.

The most prevalent NTS type of *T. rubrum* isolated in the countries such as the United Kingdom and Brazil is type 1 (10,12). Similarly, the most frequently confirmed NTS type in our study was type 1. On the contrary, an epidemiological study conducted by Jackson et al. in 2000 revealed that most of the NTS types of *T. rubrum* derived from the Japanese is a type other than type 1, and only one out of 11 isolates belong to type 1 (10). There is a difference between our research results and those of the preceding study (Jackson et al.). Despite the rapid globalization in Japan, a molecular epidemiological study on *T. rubrum* has not been carried out since 2000 (10), and there is a great difference between their study period and ours. Due to the globalization in Japan, we propose that *T. rubrum* of type 1, which is the most prevalent NTS type worldwide, has invaded Japan from all over the world. Alternatively, due to the small number of *T. rubrum* strains derived from the Japanese investigated by Jackson et al., it is possible that type 1 was not detected.

At facility A, *T. rubrum* isolates that had the same NTS type (type 1) were prevalent. On the contrary, at other facilities, various NTS types of *T. rubrum* were confirmed (types 1 to 5). Considering that many elderly people have tinea pedis (2–4), we believe that the elderly may have brought *T. rubrum* from outside of that facility. Besides, type 1, which is the NTS type of the majority of *T. rubrum* isolates collected from the elderly people residing at facility A, is the most prevalent NTS type globally (10,12). *T. rubrum* of type 1 confirmed at facility A may be the fungi acquired in other environments outside of that facility. Therefore, we concluded that the spread of *T. rubrum* only inside a long-term care facility is unlikely.

The *T. mentagrophytes* NTS type that was most frequently isolated in this study is D2II, and the number of isolates was approximately a half of the total (45%). Type D2II is the most prevalent NTS type (~40%) (11,13), and this finding is consistent with our research results. We confirmed that type D2II is the most prevalent NTS type of *T. mentagrophytes*.

*T. mentagrophytes* isolates that carried an NTS type confirmed at one facility only were collected. These *T. mentagrophytes* isolates (types C4II, F4II, and D4II) collected from several subjects residing at the same facility were not confirmed at other facilities. In addition, the prevalence rates of these types (C4II and D4II) are low worldwide (0% and 2.4%, respectively) and in Japan (1.8% and 5.0%) as well (11,13). Additionally, type F4II is the NTS type confirmed for the first time in this study. Due to the presence of *T. mentagrophytes* isolates that carried an NTS type confirmed at one facility only and were collected from several subjects at the same facility, we showed that long-term care facilities are responsible for the spread of *T. mentagrophytes*. Similarly, *T. mentagrophytes* of type D2II, which represented the majority of isolates collected at facilities B and D, may have originated from only one individual at each facility. However, type D2II is the most prevalent NTS type (11,13). To prove the mode of the spread of *T. mentagrophytes* inside such facilities, it is necessary to increase the number of facilities and subjects, and re-examination should be performed.

Although the spread of *T. mentagrophytes* at long-term care facilities is highly likely, the probability of the spread of *T. rubrum* is remarkably low. Although the reason has not been elucidated, it has been confirmed that *T. mentagrophytes* can grow in floor dust for 9 months (14). On the contrary, it has been reported that *T. rubrum* cannot survive in floor dust (14). The lifetime of *T. rubrum* and *T. mentagrophytes* is probably different; it may be a factor only *T. mentagrophytes* spreads inside a facility.

This study proved that long-term care facilities are involved in the spread of *T. mentagrophytes*. In a recent study, *Trichophyton* with low susceptibility to an antifungal drug was discovered in Japan (15), and trichophytosis may become more intractable. Keeping the living environment clean is effective as a layer of protection against dermatophyte infections, and our research results strongly indicate the importance of cleaning at a long-term care facility as a countermeasure to the spread of *Trichophyton*. This study did not elucidate the virulence factor that is involved in the spread of infection. To prevent further spread of infectious diseases such as tinea pedis and tinea unguium, additional studies should be conducted.

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**Conflict of interest** None to declare.

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