Identification of Dermatophytes by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis of Metalloproteinase-1

Ho Jung Jung1, Soo Young Kim1, Jae Wook Jung1, Hyun Jung Park1, Yang Won Lee1,2, Yong Beom Choe1,2, Kyu Joong Ahn1,2

1Department of Dermatology, Konkuk University School of Medicine, 2Research Institute of Medical Science, Konkuk University, Seoul, Korea

Background: Transgenic research on metalloprotease-1 is an emerging field in the area of plant molecular biology. The new method reported here can similarly be applied in fungal molecular biology to identify different dermatophytes. Our method is more accurate than traditional methods such as molecular analyses. Objective: To identify Trichophyton rubrum, T. mentagrophytes var. mentagrophytes, T. tonsurans, T. mentagrophytes var. interdigitale, Microsporum canis and M. gypseum, by using the restriction fragment length polymorphism (RFLP) analysis and polymerase chain reaction (PCR) to detect polymorphisms in the metalloproteinase-1 gene (MEP1). Methods: From each fungal strain, we isolated genomic DNA and performed PCR to amplify the region coding for metalloproteinase-1. Primers for the metalloproteinase-1 gene were designed based on the sequence in NCBI GenBank. Subsequently, we purified the amplified PCR product and performed RFLP analysis. After restriction enzyme digestion, BsrDI (NEB, England), the samples were subjected to electrophoresis. Four different patterns of DNA fragments were observed among 6 fungal species. Results: The DNA fragments for T. mentagrophytes var. mentagrophytes, T. mentagrophytes var. interdigitale and T. tonsurans showed similar patterns on electrophoresis and were not distinguishable, whereas T. rubrum, M. canis, and M. gypseum showed different patterns. Conclusion: To our knowledge, it is the first study to introduce the analysis of the nucleotide sequence of metalloproteinase-1 enzyme to study differentiation in dermatophytes. Based on our results, more accurate differentiation and subtyping of T. rubrum and T. mentagrophytes var. interdigitale might be possible. This might contribute to better understanding of the epidemiology and pathogenesis of dermatophyte. (Ann Dermatol 26(3) 338 ∼ 342, 2014)

Keywords: Metalloproteinases, Microsporum, Polymerase chain reaction, Restriction fragment length polymorphism, Trichophyton

INTRODUCTION

Dermatophytes are pathogenic, keratinophilic fungi that invade the stratum corneum of epidermis, hair, fingernails, and toenails of animals. Throughout the world, 43 species of dermatophyte fungi have been identified. Among these, 20 are known to infect humans and other animals. Trichophyton mentagrophytes var. mentagrophytes, T. mentagrophytes var. interdigitale, T. tonsurans, T. verrucosum, T. schönleinii, T. rubrum, Microsporum canis, M. gypseum, and Epidermophyton floccosum have been reported in South Korea. Of the nine species, T. rubrum is the most abundant and accounts for 85% to 90% of all reported cases of infection. Accurate differentiation of dermatophyte subspecies of T. rubrum, T. tonsurans, and T. mentagrophytes var. interdigitale is, therefore, of
identification of dermatophytes by these methods remains difficult. Therefore, other molecular techniques need to be considered. A variety of methods, including mitochondrial DNA restriction fragment length polymorphism (RFLP) pattern and chitin synthetase 1 nucleotide sequence analysis, have been used for simple, fast, and accurate identification of dermatophytes. Recently, the nucleotide sequences of internal transcribed spacer (ITS) regions that represent organism diversity have been analyzed. However, the ITS sequences of *M. canis* and *M. gypseum* are very similar, making differentiation using this method difficult. Moreover, this method requires the utilization of a variety of restriction enzymes. It has recently been reported that proteins from various organisms are damaged upon the activation of proteolytic enzymes, such as metalloproteinase-1, upon exposure to ultraviolet light or high temperatures. It was found that during the recovery of the damaged DNA sequences, slight alterations occur in the sequences. These alterations often result in speciation. Recently, transgenic research using the proteolytic enzyme metalloproteinase-1 has gained popularity in the area of plant molecular biology. This method can also be applied to the study differentiation in dermatophytes and may prove to be more accurate than other known molecular methods.

In this study, common pathogenic species of dermatophytes, *T. rubrum*, *T. mentagrophytes*, *T. tonsurans*, *M. canis* and *M. gypseum* were isolated and identified using the polymerase chain reaction (PCR)-RFLP technique. The gene coding for metalloproteinase-1 (MEP1) was analyzed for polymorphisms upon restriction digestion with BsrDI.

**Table 1.** Standard strains of dermatophytes used in our study

| Species                              | Strain          |
|--------------------------------------|-----------------|
| *Trichophyton rubrum*                | ATCC28188       |
| *T. mentagrophytes* var. *mentagrophytes* | CBS113880     |
| *T. mentagrophytes* var. *interdigitale* | IFM48155       |
| *T. tonsurans*                       | CBS109036       |
| *Microsporum canis*                  | IFM58292        |
| *M. gypseum*                         | IFM5292         |

**MATERIALS AND METHODS**

**Bacterial species**

The standard species of *T. rubrum*, *T. mentagrophytes* var. *mentagrophytes*, *T. mentagrophytes* var. *interdigitale*, *T. tonsurans*, *M. canis* and *M. gypseum* were spread evenly on Mycosel agar culture medium (Papacic digest of soybean meal 10 g, dextrose 10 g, cycloheximide 0.4 g, chloramphenicol 0.05 g and bactoagar 15.5 g), sterilized at 121°C for 15 minutes, and incubated at 34°C for 14 days (Table 1).

**Methods**

1) **DNA extraction**

To collect the samples with roots, a 0.5×0.5 cm×1 to 2 mm block of Mycosel agar with colonies was placed in a 1.5-ml eppendorf tube (e-tube) and centrifuged at 13,570×g at 4°C for 10 minutes. The samples were resuspended in 200 μl of Lysis buffer (100 mM Tris-HCl pH 9.5, 1 M KCl and 10 mM EDTA), pulv erized with a plastic pestle, and heated at 50°C for 30 minutes. Lysis buffer (200 μl) was added to the samples and they were centrifuged at 13,570×g at 4°C for 2 minutes. The floating upper layer of the resulting supernatant was transferred to a 1.5-ml e-tube and treated with 50 ng of proteinase K for 16 hours at 55°C. The samples were then incubated at 100°C for 30 minutes to inactivate the proteinase K. A mixture of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v; 400 μl) was added to the sample and centrifuged at 15,920×g at 4°C for 15 minutes. The supernatant was then transferred to a new tube. Isopropanol, the same volume as the supernatant, was added to the tube and the samples were incubated at −80°C for 1 hour. The tubes were then spun at 15,920×g at 4°C for 20 minutes. Seventy percent ice-cold ethanol was added to the samples, followed by centrifugation at 15,920×g at 4°C for 5 minutes. The samples were then dried and the DNA concentration was determined using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA)

2) **Metalloproteinase-1 primer**

For the PCR, the forward (5'-GACGGTTCTTTTGCTTG-3') and reverse (5'-ACTTACGACCGTGGGTGA-3') primers were designed based on the nucleotide sequence of metalloproteinase-1 deposited in GenBank Genebank (National Center for Biotechnology Information, Bethesda, MD, USA) (Fig. 1).

3) **Polymerase chain reaction amplification**

This study adopted colony PCR analysis to amplify the
metalloproteinase-1 gene from the extracted genomic DNA. Each PCR mixture contained 10-μl 10× reaction buffer; 100-μmol/L (each) dATP, dCTP, dGTP, and dTTP; 2.5 U Taq polymerase; 30 pmol of each primer; and 5-μl DNA template solution. Ultrapure water was added to increase the volume of the reaction mixture to 100 μl. PCR amplification was performed in a Veriti 96-Well Fast Thermal Cycler (Life Technologies, New York, NY, USA). Each reaction mixture was preheated to 94°C for 5 minutes. PCR amplification was performed for 35 cycles under the following conditions: 94°C for 1 minute; 52.8°C for 30 seconds; 72°C for 1 minute and 72°C for 7 minutes. The products were separated by electrophoresis on a 3% agarose gel. A single band of about 0.74 kilo base pairs was detected by ultraviolet (UV) visualization.

4) Purification of amplified DNA
Bands of the appropriate size were excised from the agarose gel. DNA extraction from the excised gel parts was performed using LaboPass™ Gel (Cosmo, Seoul, Korea) and PCR Clean-up kit (Cosmo) according to the manufacturer’s instructions.

5) Detection of restriction fragment length polymorphism in the metalloproteinase-1 regions
Ten micrograms of purified PCR sample was digested at 65°C for 16 hours with 10 U of restriction endonuclease BsrDI (New England Biolabs, Ipswich, MA, USA). Digested fragments were separated by electrophoresis on a 3% agarose gel.

RESULTS
We performed RFLP analysis for six isolated strains of dermatophytes (T. rubrum, T. mentagrophytes var. mentagrophytes, T. mentagrophytes var. interdigitale, T. tonsurans, M. canis, and M. gypseum). From the DNA fragments obtained after BsrDI restriction digestion, we were able to distinguish four different patterns (Table 2). The DNA fragment sizes in each pattern were as follows: T. rubrum, 351/216 base pair (bp); T. mentagrophytes var. mentagrophytes, 321/216/34 bp; T. mentagrophytes var. interdigitale, 320/216/34 bp; T. tonsurans, 325/216/34 bp; M. canis, 321 bp; and M. gypseum, 428/321/31 bp. Fragments from T. mentagrophytes var. mentagrophytes, T. mentagrophytes var. interdigitale, and T. tonsurans showed an identical pattern because the differences between them were of the order of 1 to 5 bp, which cannot be distinguished by electrophoresis. In addition, T. rubrum, M. canis, and M. gypseum showed their own three distinct patterns (Fig. 2).
DISCUSSION

Dermatophytes are a major cause of frequent fungal infections. In in vitro conditions, morphology of dermatophytes can easily change depending on the culture conditions. Classification of dermatophytes based on their phenotypes is not systematic. Since the 1980s, molecular classification techniques have been developed to categorize these fungi more accurately. While mitochondrial DNA RFLP analyses (using restriction endonucleases) as well as nuclear ribosomal RNA gene analyses (using nucleic acid sequences) have been attempted, some species exist that cannot be distinguished using such methods. Since the mid-1990s, analysis of diverse ITS sequences began. Summerbell et al. performed the ITS region analysis of *T. rubrum* and similar strains. They found that *T. rubrum*, *T. raubitschekii*, *T. fischeri*, and *T. kanei* share similar sequences, and that *T. soudanense* and *T. megninii* have nearly identical sequences. Furthermore, several researchers performed an ITS sequence analysis using BsuRI, HinfI, Ddel, and Mval, and they were able to differentiate 13 strains, including *T. rubrum*, *M. canis*, and *T. mentagrophytes*. Various types of proteases such as metalloproteinase-1, metalloproteinase-2, metalloproteinase-9, and metalloproteinase-12 have been previously reported. The use of metalloproteinase DNA sequences for molecular analysis of dermatophytes might lead to improvements in the understanding of their epidemiology and pathogenesis. Furthermore, our current method of categorizing dermatophytes can contribute to more efficient genotyping analysis.

ACKNOWLEDGMENT

This work was supported by Konkuk University.

REFERENCES

1. Okeke CN, Tsuboi R, Kawai M, Hiruma M, Ogawa H. Isolation of an intron-containing partial sequence of the gene encoding dermatophyte actin (ACT) and detection of a fragment of the transcript by reverse transcription-nested PCR as a means of assessing the viability of dermatophytes in skin scales. J Clin Microbiol 2001;39:101-106.
2. Gräser Y, el Fari M, Presber W, Sterry W, Tietz HJ. Identification of common dermatophytes (Trichophyton, Microsporum, Epidermophyton) using polymerase chain reactions. Br J Dermatol 1998;138:576-582.
3. Harmen D, Schwinn A, Bröcker EB, Frosch M. Molecular differentiation of dermatophyte fungi. Mycoses 1999;42:67-70.
4. Turenne CY, Sanche SE, Hoban DJ, Karlowsky JA, Kabani...
AM. Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. J Clin Microbiol 1999;37:1846-1851.

5. Summerbell R, Kane J. Laboratory handbook of dermatophytes: a clinical guide and laboratory handbook of dermatophytes and other filamentous fungi from skin, hair, and nails. Belmont (CA): Star Publishing, 1997:103-107.

6. Liu D, Pearce L, Lilley G, Coloe S, Baird R, Pedersen J. PCR identification of dermatophyte fungi *Trichophyton rubrum*, *T. soudanense* and *T. gourvili*. J Med Microbiol 2002;51:117-122.

7. Liu D, Coloe S, Baird R, Pedersen J. Application of PCR to the identification of dermatophyte fungi. J Med Microbiol 2000;49:493-497.

8. Gräser Y, Kuijpers AF, Presber W, De Hoog GS. Molecular taxonomy of *Trichophyton mentagrophytes* and *T. tonsurans*. Med Mycol 1999;37:315-330.

9. Jackson CJ, Barton RC, Evans EG. Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. J Clin Microbiol 1999;37:931-936.

10. Summerbell RC, Haugland RA, Li A, Gupta AK. rRNA gene internal transcribed spacer 1 and 2 sequences of asexual, anthropophilic dermatophytes related to *Trichophyton rubrum*. J Clin Microbiol 1999;37:4005-4011.

11. Makimura K, Tamura Y, Mochizuki T, Hasegawa A, Tajiri Y, Hanazawa R, et al. Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J Clin Microbiol 1999;37:920-924.

12. Kwon-chung KJ, Bennett JE. Medical mycology. Philadelphia: Lea and Febiger, 1992:301-311.

13. Liu D, Coloe S, Baird R, Pedersen J. PCR identification of *Trichophyton mentagrophytes* var. *interdigitale* and *T. mentagrophytes* var. *mentagrophytes* dermatophytes with a random primer. J Med Microbiol 1997;46:1043-1046.

14. Kim JA. Molecular biological approaches to the study of dermatophytes. Korean J Med Mycol 2002;7:1-5.

15. Shin JH, Sung JH, Park SJ, Kim JA, Lee JH, Lee DY, et al. Species identification and strain differentiation of dermatophyte fungi using polymerase chain reaction amplification and restriction enzyme analysis. J Am Acad Dermatol 2003;48:857-865.