A Thesis

For the Degree of Master of Veterinary Medicine

Rapid and direct detection of *Microsporum canis* from canine hairs by PCR and PCR-RFLP

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2007. 2

Abstract

Rapid and direct detection of *Microsporum canis* from canine hairs by PCR and PCR-RFLP

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Microsporum species are the most common agent of fungal dermatitis in dogs and cats and are transmitted to humans. The diagnosis of the disease is almost achieved by fungal culture and microscopic examination. But these methods take about two weeks to identify them. In this study, we amplified a part of the dermatophyte gene by PCR using TR, ITS, CHS1, dDP, MCN primer pairs. The result showed DNA concentration was increased depends on a number of hair. Each DNA sample was amplified with the primer sets corresponding to specific region of the *Microsporum canis* (TR; 580 bp, *ITS*; 720 bp, CHS1; 620 bp, dDP; 2380 bp, MCN; 639 bp) and restriction enzyme analysis (RFLP) of amplified products (TR, ITS, MCN) by *Sac*I, *Sma*I, *Eco*RⅡ was done to identify species. The PCR products amplified from the DNA revealed each band corresponding to the respective amplified region of each fungus. All of 24 dermatophytosis patients were infected with *Microsporum canis.* The nucleotide sequencing on the PCR products (ITS, CHS) were performed and completely coincided with the reference sequences of the *Microsporum canis*.

The PCR assay is quick and simply to directly detect *Microsporum canis* from the infected hair. This method is provide a sensitive and rapid detection and identification system for dermatophyte species, which may be applied to epidemiological surveys and routine practice.

Key Words: Dermatophytosis, *Microsporum canis*, PCR, PCR-RFLP

CONTENTS

Ⅰ. INTRODUCTION

The dermatophytes including *Trichophyton* spp, *Microsporum* spp, *Epidermophyton* spp, have the ability to invade keratinized structures, such as the superficial cornified skin layers, hair, nails of human and other animal, causing a superficial cutaneous infection [Kanbe *et al*, 2003]. Dermatophytosis is the most common fungal disease in small animal clinic and sometimes transmitt to human [Brilhante *et al*, 2005].

Dermatophytosis of dogs and cats is related with *Microsporum canis, Microsporum gypseum* and *Trichophyton mentagrophytes. Microsporum canis* is found in 70%, *Microsporum gypseum* in 20 % and *Trichophyton mentagrophytes* in 10% of the canine dermatophytosis cases. In cats, 98 % of the dermatophytosis case is caused by *Microsporum canis* [Rochette *et al*, 2003].

Routine identification procedures for Dermatophyte species rely on examination of the colony (shape, texture, and rate of growth) and microscopic morphology (size and shape of macroconidia and microconidia, spirals, nodular organs, and pectinate branches). Further identification characteristics includes nutritional requirements (vitamins and amino acids) and temperature tolerance, as well as urease production, alkaline production of bromocresol purple medium, in vitro hair perforation, etc [Weitzman *et al*, 1995]. Morphological identification is made difficult by the strict similarity among cells belonging to fungi from related genera and long time required for identification [Turin *et al*, 2000]. According to many author [Makimura *et al*, 1999; Faggi *et al*, 2001], dermatophytes in culture may easily loose their classical morphological features. Morphological and physiological characteristics can frequently vary; in fact, the phenotypic features can be easily influenced by outside factor such as temperature variation, medium, and chemotherapy. Therefore fungal identification is often difficult [Liu D *et al*, 2000]. Molecular methods, such as restriction fragment length

polymorphism analysis of mitochondrial DNA [Jackson *et al*, 1999; Kanbe *et al*, 2003; Kamiya *et al*, 2004; Shin *et al,* 2003], sequencing internal transcribed spacer (ITS) region of the ribosomal RNA [Gräser *et al*, 1999], sequencing protein-encoding genes [Kano *et al*, 1997], polymerase chain reaction (PCR) and random amplification of polymorphic DNA (RAPD) [Brilhante *et al*, 2005; Kim *et al*, 2001], arbitrarily primed PCR (AP-PCR) [Liu *et al*, 1997; Liu *et al*, 2000], and PCR fingerprinting [Faggi *et al*, 2001] have brought important progress in distinguishing between species and strains. PCR technology is simple, rapid, and in the absence of specific nucleotide sequence information for the many dermatophyte species, able to generate species-specific or DNA polymorphisms on the basis of characteristic band patterns detected by agarose gel electrophoresis [Liu *et al*, 2000]. PCR and PCR-RFLP techniques targeting the DNA topoisomerase II gene are available as a tool for the identification of the major dermatophyte species because of it rapidity and simplicty [Kanbe *et al*, 2003; Kamiya *et al*, 2004]. By PCR analysis with the primer pair specific to the chitin synthase 1 (CHS*1*) gene of dermatophytes, dermatophyte DNA could be diagnosed directly and rapidly in clinical skin samples [Kano *et al*. 2003]. Also, the sequence analysis of CHS*1* gene is very useful for understanding the evolution of dermatophyte species [Hirai *et al*, 2001]. Three primer pairs (TR1-TR2, B2F-B4R, ITS1-ITS4), amplifying fragments of the highly conserved gene coding for small ribosomal RNA (18S rDNA) and the adjacent internal transcribed spacer (ITS) rDNA, these primer sets, in single amplification or double-rounded PCR assays, allowed specific amplification when applied to a wide number of fungal DNA from human and animal tissue specimen, including dermatophytes (genera *Trichophyton, Microsporum*), several yeast species (*Candida, Saccharomyces, Cryptococcus, Malassezia*) and moulds (*Aspergillus, Penicillium*) [Turin *et al*, 2000].

In this study, canine dermatophytosis with *Microsporum. canis* diagnosed by

PCR and PCR-RFLP analysed using the DNA extracted from hair samples of dogs.

Ⅱ. MATERIALS AND METHODS

1. Sample collection

In order to develope polymerase chaine reaction (PCR) method for rapid and direct detection of *Micosporum canis* from infected dog hairs, samples were collected from 24 fungal dermatophytosis suspected dogs submitted to the Animal Hospital of the Cheju National University as well as animal clinics in Seoul region. Dermatitis caused by bacteria, ectoparatite and other factors have been excluded by a preliminary examination, such as hair plunking, skin scraping, and wood's lamp examination.

2. Isolation and examination of fungal agents

Hairs of intact skin lesions were inoculated onto Sabouraud dextrose agar (SDA; Difco, MD, USA), Dermatophyte test medium (DTM), and Potato dextrose agar (PDA; Difco, MD, USA) and the plates were incubated at 25℃ until 2 weeks. Texture and surface of the colony and the presence or absence of pigmentation were observed. The morphology of the fungal colony was examined, and haphae and macroconida formations were observed by microscope after lactophenol cotton blue staining.

3. Standard strain

Standard strain obtained from used in this experiment is *Microsporum canis* ATCC 52067 purchased from the American Type Culture Collection.

4. Extraction of fungal DNA

1) Extraction of DNA from cultured fungi

Fungal genomic DNA was prepared using a rapid method described earlier [Turin *et al*, 2000]. Fungal colony was suspended on the culture plate with 1-2 ㎖ of TE buffer (1 mM EDTA, 10 nM Tris-HCl, pH 8.0). The colonies were scrapped and then added to sterile Eppendorf tubes. The suspension was centrifuged at 7,500 X g for 5 min, removed the supernatant and then resuspended into 400 ㎕ of Tris-based buffer (75 mM NaCl, 25 mM EDTA, 50 mM Tris-HCl, pH 8.0) containing 1 ㎕ of lyticase (15 U/㎕) by vigorous vortexing. The suspension was incubated at 30℃ for 20 min to allow to activate lyticase, and then treated with 10 % sodium dodecyl sulphate (SDS) 100 ㎕ and 20 ㎕ of proteinase K (2 U/㎕), pulverized to pellet pestle. After reaction at 56℃ for 1 h to activate proteinase K, same amount of PCI (Phenol : Chloroform : Isoamylalchol = 25 : 24 : 1) were added to the reaction mixture, and DNA was segregated by centrifugation at 7,500 X g for 5 minute. Subsequently, the DNA was precipitated with the same amount of isopropanol and rinsed once with 70% ethanol. The DNA pellet was resuspended in TE buffer. DNA concentration was finally measured using spectrometor (GeneQuant).

2) DNA extraction from infected hair

To extract DNA from hair of dermatophytosis suspected dog, 1 to 5 hairs on lesions were put into a Plain Capillary tube (Marienfeld, Germany), and injected 10 ㎕ of cocktail buffer composed of Tris-based buffer (400 ㎕), lyticase (15 U/㎕), 10% SDS (100 ㎕), and proteinase K (2 U/㎕). One side of the Plain Capillary tube containing hair was sealed, and then centrifuged to allow the hair to sink into the buffer. For the activation of both lyticase and proteinase K, the tube were reacted for 1 h at 56℃ following incubation for 20 min at 30℃. Both enzymes were inactivated by incubation for 2 min at 95℃ to prevent PCR disturbing. The extracted solution into the capillary tube was carefully transferred into PCR tube and then used as PCR template.

5. PCR

1) Primer sets

Primer sets for detection of *M. canis* infection from canine hairs were TR1, TR2 specific to 18S rRNA and ITS1, ITS4 specific to 5.8 S rRNA published by Turin *et al* (2000), and CHS1 primer pair specific to Chitin synthase 1 gene, published by Kanbe *et al* (2000). Also following Kanbe *et al* (2003), dermatophyte factor has been identified by using common primers (dDP F2, dDP R2) and specific primers (MCN F2, MCN R2) of dermatophyte DNA topoisomerase 2 genes. To confirm DNA which extracted from hair, following Dakhama *et al* (1996) report β-actin primer sets of β-actin genes has been used (Table 1). Six pairs of specific were custom synthesized by Bioneer Korea.

Table 1. Primer sets designed on the conserved sequences of the 18S rRNA, 5.8S rRNA, DNA topoisomerase Ⅱ and canine β-actin genes.

Primer	Tm (℃)	Sequence (5'-3')	Size (bp)	Reference
TR 1	50	GTTTCTAGGACCGCC	580	Turin *et al*
TR 2		CTCAAACTTCCATCGACTTG		(2000)
ITS 1	50	TCCGTAGGTGAACCTGCGG	720	Turin *et al*
ITS 4		TCCTCCGCTTATTGATATG		(2000)
CHS1 F	50	CTGAAGCTTACKATGTAYAAYGARGAY	620	Kanbe *et al*
CHS1 R		GTTCTCGAGYTTRTAYTCRAARTTYTG		(2000)
dDP F2	58	GTYTGGAAYAAYGGYCGYGGTATTCC	2380	Kanbe *et al*
dDP R2		RAAVCCGCGGAACCAKGGCTTCATKGG		(2003)
MCN F2	58	GCTGGTAAATAACACCGATGATGG	639	Kanbe *et al*
MCN R2		TGTATCTGATATGCATACCTTCC		(2003)
β-actin F	50	CGTTGCTATCCAGGCTGTGCTAT	435	Dakhama *et al*
β-actin R		GTAGTTTCGTGGATGCCACAGGA		(1996)

R; A or G, Y; C or T, K; G or T

2) PCR condition and agarose gel electrophoresis

For the PCR amplification, a reaction mixture was contained 1 ㎕ (100 ng/㎕) of template DNA, 2 ㎕ of 10X PCR buffer (2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl), 0.5 ㎕ of 2.5 mM dNTPs (dATP, dGTP, dCTP, dTTP, 2mM each), 0.2 ㎕ of Taq polymerase (5 U/㎕, Takara, Japan), 1 ㎕ of primer (10 pmol/㎕) and distilled water 14.3 ㎕. Takara PCR Thermal Cycler (Takara Biomedicals Co. Japan) has been used for PCR.

The PCR cycle parameters were as follows: initial denatureration at 95℃ for 5 min; then 35 cycles at 95℃ for 30 s, 50℃ for 30 s and 72℃ for 30 s using β-actin, ITS, TR, CHS1 primer sets, and annealing condition was at 58℃ for 20 s in case of other primer sets, such as dDP F2, dDP R2, MCN F2, and MCN R2. PCR products were analyzed using agarose gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.0) at 100 V for 30 min in a gel composed of 1.8% (w/v) agarose (SEA KEM, FMC, USA). The

gels were stained with 0.5 ㎍/ml of ethidium bromide and then destained at room temperature for 30 min in distilled water. The DNA bands was visualized with a UV tansilluminator (Uvitec, UK) and photographed.

6. Sensitivity of PCR amplification

Serial dilutions (100 ng to 100 fg) of the *M. canis* genomic DNA were prepared in order to estimate the sensitivity of the PCR amplification using MCN primer set.

7. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

For RFLP analysis, the PCR products amplified by each primer sets were purified using a Geneclean® II Kit (Q-bio gene, USA) from each PCR solution, and used as the substrates for restriction enzymes. TR1-2, ITS1-4 and MCN F-R PCR products were digested with *Sac*I (Takara, Japan) and *Eco*RⅡ (Takara, Japan), *Sma*I (Takara, Japan) and *EcoR*Ⅱ, and *EcoR*Ⅱ, respectively, for 2 hour at 37℃ in a total volume of 10 ㎕ containing 4 ㎕ of the specific PCR product and 1 ㎕ of 10X buffer and 1 ㎕ of restriction enzyme (10 U/㎕). The digested samples were analyzed on 1.8% agarose gel (SEA KEM, FMC, USA) and then visualized by UV transilluminator following ethidium bromide staining.

8. Sequencing of PCR products.

For confirmation of the specificity of the primer sets, the DNA fragments generated by ITS1-4 and CHS1 primer sets were sequenced. After agarose gel

electrophoresis, DNAs were purified using Geneclean® II Kit (Q-bio gene, USA) and submitted and analyzed by SolGent (Korea) company.

Ⅲ. RESULTS

1. Isolation and identification of dermatophytes from clinical samples

M. canis was isolated from all 24 fungal infected samples estimated by the preliminary examination. Fungal isolates were identified by traditional morphological observations of giant colonies and macroconidia of hyphae using a light microscope. The colony morphology of *M. canis* growing onto SDA and PDA was characterized by white cottony to woolly like apparence. The color of Dermatophyte test medium changed yellow to red. A number of fusiformis macroconidia surrounded with thick wall were observed from the slide preparation staining with lactophenol cotton blue (Figure 1).

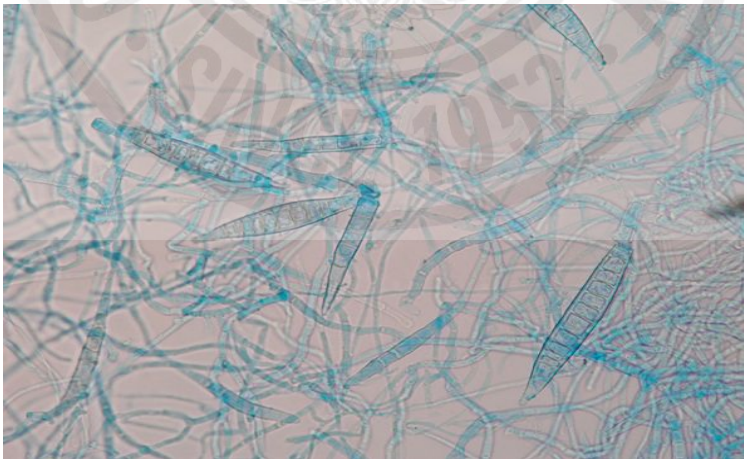

Figure 1. Macrocornidia morphologies of dermatophytes Slide preparation was stained the fungal culture at 25℃ for 2 weeks with lactophenol cotton blue (X 400).

2. Direct detection of *M. canis* from hair

PCR technique using the fungal DNA extracted directly from infected hair allowed to identify the dermatophytosis by *M. canis* within 6 hours. The amount of total DNA extracted from 1, 2, 3, 4 and 5 infected hairs was 64.4 ± 20.66, 123.3 ± 37.14, 134.2 ± 20.10, 156.4 ± 63.19, and 202.2 ± 19.30 ㎍/㎕, respectively (Table 2).

Table 2. DNA concentration according to the number of hair

Number of hairs	DNA Concentration (㎍/㎕)
1	64.4 ± 20.66
2	123.3 ± 37.14
3	134.2 ± 20.10
4	156.4 ± 63.19
5	202.2 ± 19.30

Each primer pairs produced the expected size of PCR products, such as 2380 bp (dDP F2, dDP R2), 639 bp (MCN F2, MCN R2), 580 bp (TR1, TR2), 720 bp (ITS1, ITS4), and 620 bp (CHS 1F, CHS 1R) from both Microsporum canis ATCC 5206 standard strain and the dermatophyte isolates (Figure 2).

3. Specificity of the PCR amplification

The genomic DNA purified from the colonies of *M. canis* was amplified by MCN-F2 and MCN-R2, in oder to determine the sensitivity of the PCR. The intensity of the major band generated from the serial diluted DNA samples derived from 100 ng to 100 pg and some faint bands were generated along with the major band in 100 ng of the DNA, but these were clearly less prominent than the major band (Figure 3).

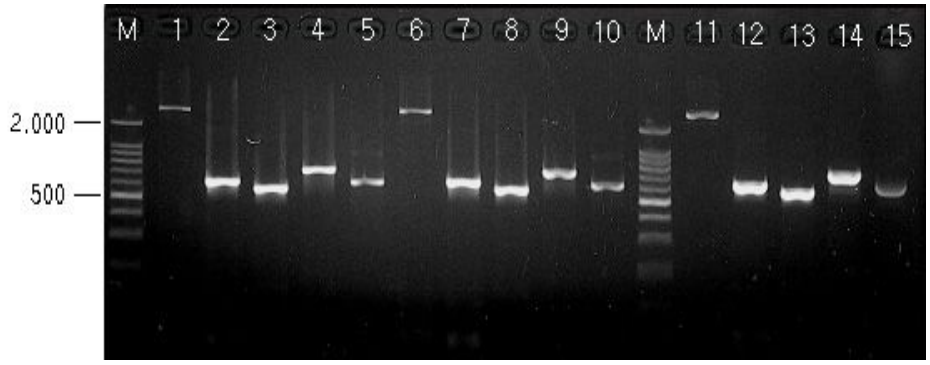

Figure 2. PCR amplification of genomic DNA samples was carried out with dermatophyte specific primers. Lane M indicates 100 bp ladders (Bioneer, Korea); Lanes 1-5, lanes 6-10 and lanes 11-15 are PCR products of Microsporum canis ATCC 52067, dermatophytosis patient 1 and dermatophytosis patient 2, respectively; Lanes 1, 6 and 11, dDP (2380 bp); Lanes 2, 7 and 12; MCN (639 bp); Lanes 3, 8 and 13, TR (580 bp); Lanes 4, 9 and 14, ITS (720 bp); Lanes 5, 10 and 15, CHS1 (640 bp). The number on the left side of panel indicate the size (bp) of the PCR product.

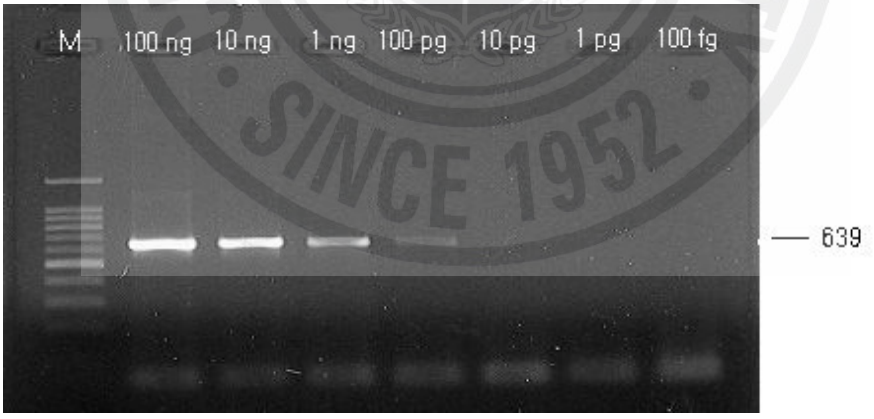

Figure 3. Sensitivity of the PCR amplification using MCN F2 and MCN R2. PCR products were generated from different amounts of the purified genomic DNA of *Microsporium canis* ATCC 52067. Lane M indicates 100 bp DNA ladders (Bioneer). Lane 1-7 indicate the concentration of template DNA purified from *M. canis*. The number on the right side of panel indicate the size (bp) of the PCR product.

4. Specificity of PCR amplification compared with other diagnostic methods

All 24 dogs were diagnosed not to be infected by bacteria, ectoparasites, and other factors through the preliminary examination, and were observed by the traditional diagnostic methods, such as the wood's light examination, the microscopic examination, and the fungal isolation method, in order to evaluate the fungal infection. The direct PCR amplification showed that all 24 dogs have been infected with *M. canis,* whereas the fungal isolation method was fail to isolate *M. canis* from 5 dogs (21%), the wood's light and the microscopic examination indicated that respectively 10 (42%) and 6 (25%) dogs were infected with fungi.

Table 3. Comparison of results obtained from the infected dog hairs analyzed by using wood's light, microscopic, isolation, and PCR method.

No. of samples	No. (%) of samples positive for *M. canis* in			
	Microscopic test	Wood's light	Isolation method	PCR
24	6 (25)	10 (42)	19 (79)	24 (100)

4. Identification of *M. canis* by PCR-RFLP

PCR-RFLP is useful for identification of dermatophyte species after PCR amplification. The PCR products amplified by each primer sets were purified and used as substrates for the RFLP analysis using *Sac*I, *Sma*I or *Eco*RⅡ As expected, 2 (383 and 197 bp), 3 (452, 173 and 95 bp) and 4 (320, 201, 96 and 22 bp) fragments were respectively generated from the DNA substrates amplified from TR1-TR2, from ITS1-ITS4 and from MCNF-MCNR primer pairs after

digestion with *Eco*RⅡ (Table 4 and Figure 4). The PCR products generated from TR1-TR2 and ITS1-ITS4 primer pairs were digested as 2 (424 and 156 bp) and 3 (530, 99 and 91 bp) by *Sac*I and *Sma*I, respectively (Table 4 and Figure 4). The PCR products from all 24 samples showed same polymorphism by RFLP analysis, indicating that these were coincided with the fragments generated from *M. canis* (Table 4).

Table 4. List of the expected sizes of DNA fragments in PCR-RFLP using *Sac*I, *Sma*I and *Eco*RⅡ of PCR products from *Microsporum canis*.

Primer set	Enzyme	DNA fragment (bp)
TR1-TR2	*Sac*I	424, 156
	*Eco*RⅡ	383, 197
*ITS*1-*ITS*4	*Sma*I	530, 99, 91
	*Eco*RⅡ	452, 173, 95
MCN F2-MCN R2	*Eco*RⅡ	320, 201, 96, 22

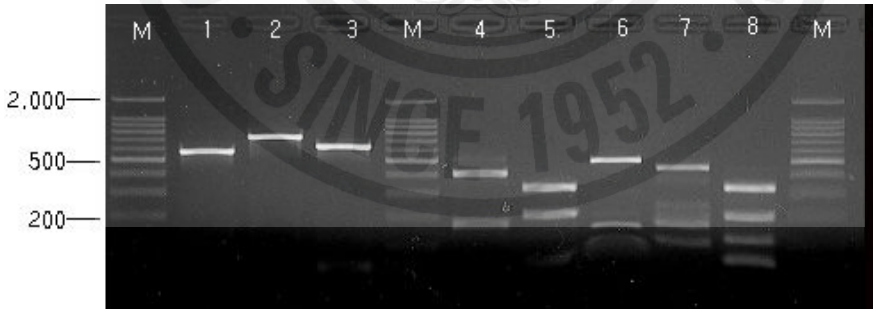

Figure 4. RFLP obtained from PCR products of *Microsporum canis* ATCC 52067 digested with *Sac*I, *Sma*I and *Eco*RⅡ. Lane M indicates 100 bp DNA ladders (Bioneer). Lane 1, TR 1-2 (580 bp), Lane 2: ITS 1-4 (720 bp), Lane 3: MCN F-R (639 bp), Lane 4: TR- *Sac*I (424, 156 bp), Lane 5: TR- *Eco*RⅡ (383, 197 bp), Lane 6: ITS-*Sma*I (530, 99, 91 bp), Lane 7: ITS- *Eco*RⅡ (452, 173, 95 bp), Lane 8: MCN-*EcoR*Ⅱ (320, 201, 96, 22 bp).

5. Sequence analysis

Nucleotide sequence analysis of the ITS1 gene fragments indicated coincidental results of *M. canis* (GeneBank accession no. AB193642), reported in Yarita *et al,* (2004) and Nucleotide sequences of the CHS1 gene indicated coincidental results of *Arthroderma otae,* reported in Kano *et al* (GeneBank accession no. AB003563).

Ⅳ. DISCUSSION

The morphology of Dermatophytes is various depending on culture conditions. Especially, typical morphology of Dermatophytes might be changed in a case of long time subculture due to villous denaturation [Makimura *et al,* 1999; Faggi *et al,* 2001]. Therefore, it is difficult to identify Dermatophyte species by the traditional morphological examination. Several methods have been used for identification of the fungal agents, such as urease production examination and hair perforation. But these methods require long time and sometimes, fail to identify the agent [Kim *et al*, 2002]. Dermatophytes examination method used most common, KOH smear examination is useful to observe fungal hyphae in view of cost, time consuming, and simplicity but there are lots of difficulties of clinical application because its diagnostic sensitivity is very low. It is essential to isolate an agent for diagnosis of fungal disease, but the agent is isolated from 20-50% of cases due to interference of bacterial contamination in samples. Other than KOH smear test or fungal culture method, immunohistochemisty and flow cytometry have been researched for more accurate diagnosis [Pierard *et al,* 1996]. But monoclonal antibody specific to each fungus is essential in immunohistochemistry method, and standard reference values on all fungal agents are able to utilize for flow cytometry.

Recently molecular biological techniques have been used for more accurate and fast diagnosis of diseases, covering the disadvantages of common diagnostic methods as mentioned above. Molecular biological technique using PCR is well known as the most sensitive and specific methods to detect the fungal agents, even in the existence of small amount of fungal genetic element.

Sensitivity examination of PCR from internal transcribed spacer (*ITS*) 1 part of *Trichophyton tonsurans,* concentration was amplified to 10 pg [Yoshida *et al,* 2006], and at Chintin Synthase (*CHS*) 1 part, PCR sensitivity examination of *M.*

canis (VUT-77054) concentration was amplified to 1 pg [Kano *et al,* 2003].

In this study, result of sensitivity measurement of *M. canis* DNA PCR from DNA topoisomerase Ⅱ part, amplifed concentration to 100 pg was checked using MCN primer sets. However, dermatophytosis molecular biology based technique, first of all, pure separate lesion region fun without denaturation. There are many protocols to extract the fungal DNA, but most of these methods take long time and require large amount of tissue sample.

The integrity of the fungal cell wall and membrane were compromised by using classic method to isolate and purify the DNA from cell and environmental debris [Bever *et al*, 2000]. However, typical method of isolate and purify DNA is to damage integrity cell wall and cell membrane of fungi [Elasa *et al*, 2000].

As well as, there is a extract method using isopropanol and ethanol but this method has a weak point of losing DNA in purification case when to separate small amount of DNA from fungi cell. Another method, Homogenization uses glass-bead-beating or a similar type of the cell lysis matrix to extract fungal DNA [Smit *et al*, 1999]. For PCR/sequencing application, the Qiagen DNeasy Plant Tissue Kit protocol was used to purify the DNA. Using a simple yet rapid and reliable assay, the method allowed batch DNA extraction from multiple fungal isolates. Previously, specialized instrumentation was required, and was time-consuming or not conducive to batch process. However, use of this assay will allow researchers to obtain DNA from fungi quickly for use in molecular [Griffin *et al*, 2002].

In the experiment, we used lyticase and proteinase to extract DNA of fungi and fungal DNA was directly extracted from infected hair within 2 hour. This fungal DNA was extracted also directly extracted DNA from dermatophyte by infected hair, and PCR has been operated.

Based on the genomic sequences of the DNA topoisomerase II genes of the dermatophytes, the major dermatophyte species, a common primer set (dPsD1) in these species and species-specific primer sets (PsT and PsME) of each species were designed for PCR-based identification. As well as tested for their

specificities in PCR amplifications.

The method consisted of amplification of the genomic DNA topoisomerase II gene by the common primer set, followed by a second PCR with the primer sets consisting of species-specific primers for each dermatophyte species. PCR and PCR-RFLP techniques are rapid, simple and is available as a tool for identification of the major dermatophyte species targeting the DNA topoisomerase II [Kanbe *et al*, 2003; Kamiya *et al*, 2004]. Dermatophyte DNA could be diagnosed directly and rapidly in clinical skin samples by PCR analysis with the primer pair specific to the chitin synthase 1 (*CHS*1) gene of dermatophytes [Kano *et al*, 2003].

Three primer pairs (TR1-TR2, B2F-B4R, ITS1-ITS4), amplifying fragments of the highly conserved gene coding for small ribosomal RNA (18S rRNA). Also the adjacent internal transcribed spacer (*ITS*) rDNA. In single amplification or double-rounded PCR assays, when applied to a wide number of fungal DNA from human as well as animal tissue specimens, including dermatophytes (genera *Trichophyton, Microsporum*), several yeast species (*Candida, Saccharomyces, Cryptococcus, Malassezia)* and moulds (*Aspergillus, Penicillium*), these primer sets allowed specific amplification [Turin *et al*, 2000].

Following Kanbe *et al* (2000) report, CHS1 primer pair of Chintin synthase 1 gene and Turin *et al*. (2000) report, TR primer pair inner and 18S rRNA, *ITS*1 primer pair inner 5.8S rRNA were used in these experiments. These primers are useful for dermatophyte identification. Also using common primer set (dPsD1) as well as species specific primer sets (PsT and PsME) about Kanbe *et al* report followd by DNA topoisomerase II genes, agent identification of dermatophytes were activated. Result of PCR all DNA product which extracted in this experiment and are indetified with *Microsporum canis* infection.

Ribosomal RNA of eukaryotic cell, conserved sequence in all cell is existing and depends on phylogenetic individual, conserved sequence, which commonly show, starts to evolute, variable sequence which change formed in recent molecular biology experiment is most used. Recently, fungal phylogenetic

analysis as well as for identification, *ITS*1 part located between 18S rRNA and 5.8S rRNA in ribosomal RNA part is far most used [Kano *et al*, 2003; Turin *et al*, 2000; Choi *et al*, 2000]. The sequence analysis of *CHS* I gene is very useful for understanding the evolution of dermatophyte species [Hirai *et al*, 2001; Kim *et al*. 2002].

Restriction fragment length polymorphisms is analysis of the *NTS* and *ITS* intergenic regions of the rDNA repeat, it is a valuable technique for both molecular strain differentiation of *T. rubrum* and species identification of common dermatophyte fungi. The contiguous ITS and 5.8S rRNA regions were amplified from 17 common dermatophyte by using the universal primers ITS 1 and ITS 4. Digestion of the amplified ITS products with the restriction endonuclease *Mva*I produced unique and easily identifiable fragment patterns for a majority of species [Jackson *et al*, 1999].

In the present study, the PCR-RFLP technique did not detected polymorphism among the analysed *M. canis* samples. In the reaction, regions of DNA topoisomerase II gene was amplified. Based on the reaction, identical amplicon and profiles were seen when treated with enzymes *Sac*I, *Sma*I, *Eco*RⅡ. This research did not observe any kind of polymorphism among the strains, in spite of having an ample phenotype variation.

Nucleotide sequence of the *ITS*1 and *CHS*1 gene fragments generated from PCR of hair samples were coincident with that of *M. canis* reported in Yarita *et al,* (2004) and of *Arthroderma otae* reported in Kano *et al* (1997), respectively. This result indicates that *M. canis* strain infected in dogs of Korea might be similar to that of other countries on the base of partial sequence of CHS1 gene, which is useful for understanding the evolution of dermatophyte species [Hirai *et al*, 2001]. The PCR assay is quick and simple to detect *Microsporum canis* directly from the infected hair. This method provides sensitive, rapid detection and identification for dermatophyte species, which may be applied to epidemiological surveys and routine practice.

Ⅴ. CONCLUSION

It was possible to extract fungal gene directly from dermatophytosis patient hair by DNA extraction methods in this experiment. By this method, using primer set which is useful of dermatophytosis examinations to extracted DNA, it has been checked that each DNA band is amplified through PCR and required around 6 hours in this experiment.

The intensity of the major band generated from the serial diluted DNA samples derived from 100 ng to 100 pg and some faint bands were generated along with the major band in 100 ng of the DNA, but these were clearly less prominent than the major band.

The restriction enzymes *Sac*I, *Sma*I and *Eco*RⅡ digested the amplified lesions and cut the amplicon lesions into two, three or four segments with the below. Despite the differing section profiles among the enzymes, the phenomena of polymorphism was not observed in any of the samples.

As following, by extracting DNA from hair product in short time, it was possible to examine infection of dermatophyte more accurate and fast. Also this can higher the percentage of dermatophytosis examination, accurate incidence rate or distribution of agent strains etc. It is expected to give lots of support to experiment of dermatophytosis and clinical.

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