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High Prevalence of Lipid-Dependent *Malassezia* Infections in Dogs

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Abstract : *Malassezia* (*M.*) is a member of normal mycobiota in warm-blooded vertebrates. Increased humidity is likely to be crucial in this infection. We studied the proportion of the species infected in dog during summer of Korea. Fifty samples were analyzed by PCR-RFLP and direct sequencing from June 2006 to October 2006. The study showed that lipid-dependent species was main pathogen (*M. furfur*; 86%, *M. obtusa*; 10%) while *M. pachydermatis* (4%) has only small portion. This result suggests that *Malassezia* infection has endemic characters that can be affected by the climate (temperature and humidity) in dogs.

Key words **:** lipid-dependent species, *Malassezia* infection, dog.

Introduction

Malassezia is a member of normal mycobiota in warm-blooded vertebrates. Thirteen species of *Malassezia* (*M.*), *M. pachydermatis, M. furfur, M. globosa, M. obtusa, M. ristricta, M. sloofiae, M. yamatoensis, M. nana, M. dermatis, M. sympodialis, M. japonica, M. caprae* and *M. equina,* have been isolated (8,9,13). *M. pachydermatis*, a non-lipid dependent species, has been identified in dogs and cats as a normal microbial flora and common pathogen, while *M. furfur, M. obtusa* and *M. sympodialis* have rarely been observed (3,5,7,14).

Many predisposing factors have been hypothesized to explain the change of commensal *Malassezia* species to pathogenic species. Among these factors, increased humidity is likely to be crucial (8). In Korea, the summer is marked by hot and humid climate, so we proposed that the ratio of infection by lipid-dependent species may increase in relative proportion to the ratio of infection by non-lipid dependent species in outbreaks of *Malassezia* infection. Therefore, we studied *Malassezia* infected dogs during the summer season in the Korea by using PCR-RFLP with *Cfo*I and *Bts*CI and direct sequencing to determine the diversity of *Malassezia* species.

Materials and Methods

Clinical evaluation of outpatients

Sample collection was performed from June 2006 to October 2006. In cases which *Malassezia* infection was suspected based on the history and physical examination, a microscopic examination of tape imprint specimen was performed. After *Malassezia* infection had been confirmed, samples were collected aseptically, and fungicidal drugs were administered for treatment. Table 1 shows information for the patients selected.

Incubation

Samples were inoculated in modified Dixon's medium, containing 3.6% malt extract, 0.6% peptone, 2.0% desiccated ox bile, 1.0% Tween 40, 0.2% glycerol, 0.2% oleic acid, and 1.2% agar, pH 6.0. Inoculated plates were incubated aerobically at 37°C for 7 days and incubated plates were stored at 4°C until analysis.

Fungal DNA extraction

Malassezia DNA was extracted using modified vortexing with glass beads and Wizard genomic DNA purification kit (Promega, Madison, USA). A portion of the cultured *Malassezia* extracted DNA sample was transferred to a sterile eppendorf tube by using a sterile micropipette tip, and suspended in 300 µl phenol/chloroform/iso-amyl alcohol (Phe/Chl/IAA) (24:24:1). Cultured *Malassezia* DNA-containing eppendorf tubes were vortexed with 500 mg, acid-washed, 0.4-0.6 mm diameter glass beads (Sigma, St Louis, USA) for 30 min, at the highest setting of the vortex mixer (Genie 2, Fisher Scientific, USA). After 2 min centrifugation at 4°C and 16,000 *g*, the aqueous layer was removed and re-extracted once with an equal volume of Phe/Chl/IAA (24:24:1), and then once with an equal volume of Chl/IAA (24:1). The resulting yeast pellet was resuspended in 300 µl Nuclei lysis solution (Promega), and 100 µl protein precipitation solution (Promega) was added and vortexed for 1 min. The mixture was incubated for 5 min on ice, followed by centrifugation for 3 min at 4°C and 13,000 g. The upper, aqueous layer was transferred to a clean tube containing an equal volume of room temperature isopropanol. After 2 min centrifugation at 4°C and 16,000 *g*, the aqueous layer was discarded, and the pellet was washed with

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Table 1. Information of *Malassesia* infected dogs

No.	Signalment	Sampling site	Diagnosis	Result	No.	Signalment	Sampling site	Diagnosis	Result
1	Shitzhu, 6Y, female	Ear canal (right)	Otitis externa	*M. furfur**	26	Pekingese, 2Y, female	Facial fold	Demodicosis	*M. furfur*
2	Cocker spaniel, 6Y, male	Ear canal (left)	Atopic dermatitis	*M. furfur**	27	Tosa, 2Y, male	Ear canal (left)	Otitis externa	*M. furfur*
3	Cocker spaniel, 6Y, male	Ear canal (right)	Atopic dermatitis	*M. furfur**	28	Tosa, 2Y, male	Ear canal (right)	Otitis externa	*M. furfur*
4	Cocker spaniel, 6Y, male	Interdigit	Atopic dermatitis	*M. furfur**	29	Shitzhu, 5Y, male	Ear canal (left)	Otitis externa	*M. furfur*
5	Cocker spaniel, 6Y, male	Facial fold	Atopic dematitis	*M. furfur**	30	Shitzhu, 5Y, male	Ear canal (right)	Otitis externa	*M. furfur*
6	Shitzhu, 2Y, female	Ear canal (left)	Otitis externa	*M. furfur**	31	Pointer, 2Y, male	Ear canal (left)	Otitis externa	*M. furfur**
7	Shitzhu, 2Y, female	Ear canal (right)	Otitis externa	*M. furfur**	32	Pointer, 2Y, male	Ear canal (right)	Otitis externa	*M. furfur**
8	Mongrel, 9Y, female	Ear canal (left)	Otitis externa	*M. furfur**	33	Maltese, 3Y, male	Ear canal (left)	Otitis externa	*M. furfur**
9	Mongrel, 9Y, female	Ear canal (right)	Otitis externa	*M. furfur**	34	Maltese, 3Y, male	Ear canal (right)	Otitis externa	*M. furfur**
10	Yorkshire terrier, 6Y, female	Ear canal (left)	Otitis externa	*M. obtusa*	35	Shitzhu, 1Y, male	Ear canal (left)	Otitis externa	*M. furfur**
11	Yorkshire terrier, 6Y, female	Ear canal (right)	Otitis externa	*M. furfur**	36	Shitzhu, 1Y, male	Ear canal (right)	Otitis externa	*M. furfur*
12	Maltese, 10M, female	Ear canal (right)	Demodicosis	*M. furfur**	37	Pug, 4Y, male	Interdigit	Pododermatitis	*M. furfur*
13	Beagle, 2Y, male	Ear canal (right)	Otitis externa	*M. furfur**	38	Pug, 4Y, male	Abdomen	Dermatitis	*M. furfur*
14	Maltese, 6M, male	Ear canal (left)	Juvenile cellulitis	*M. furfur**	39	Shitzhu, 7Y, female	Ear canal (left)	Otitis externa	*M. furfur*
15	Maltese, 6M, male	Ear canal (right)	Juvenile cellulitis	*M. obtusa*	40	Maltese, 3Y, male	Ear canal (right)	Otitis externa	*M. furfur*
16	Maltese, 6M, male	Facial fold	Juvenile cellulitis	*M. furfur**	41	Shitzhu, 6Y, male	Ear canal (left)	Otitis externa	*M. furfur*
17	Yorkshire terrier, 8Y, male	Ear canal (left)	Otitis externa	*M. furfur**	42	Shitzhu, 6Y, male	Ear canal (right)	Otitis externa	*M. furfur*
18	Pointer, 1Y, male	Ear canal (right)	Otitis externa	*M. obtusa*	43	Beagle, 2Y, male	Ear canal (left)	Otitis externa	*M. pachydermatis**
19	Great pyrenees, 2Y, male	Ear canal (left)	Otitis externa	*M. furfur**	44	Beagle, 2Y, male	Ear canal (right)	Otitis externa	*M. pachydermatis*
20	Great Pyrenees, 2Y, male	Ear canal (right)	Otitis externa	*M. furfur**	45	Shitzhu, 8Y, male	Ear canal (left)	Otitis externa	*M. furfur*
21	Maltese, 10Y, male	Ear canal (right)	Otitis externa	*M. furfur**	46	Shitzhu, 8Y, male	Ear canal (right)	Otitis externa	*M. furfur*
22	Maltese, 7Y, male	Ear canal (left)	Otitis media	*M. furfur**	47	Cocker spaniel, 5Y, male	Ear canal (left)	Otitis externa	*M. furfur*
23	Maltese, 7Y, male	Ear canal (right)	Otitis media	*M. obtusa*	48	Shitzhu, 2Y, male	Ear canal (left)	Otitis exterrna	*M. furfur*
24	Miniature schnauzer, 3Y, male	Ear canal (left)	Food allergy	*M. obtusa*	49	poodle, 7Y, male	Ear canal (right)	Otitis externa	*M. furfur*
25	Mongrel, 9Y, female	Ear canal (left)	Mammary tumor	*M. furfur*	50	Maltese, 3Y, male	interdigit	Otitis externa	*M. furfur*

*Samples that were analyzed by direct sequencing.

room temperature 70% ethanol. The resulting DNA pellet was air-dried for 5 min and resuspended in 30 µl DNA rehydration solution (Promega). After adding 1.5 µl Rnase, the rehydrated DNA pellet was incubated at 37°C for 1 h, and the resulting solution was held at 4°C until further use.

PCR amplification

Primer selection was based on alignment of published 26S ribosomal DNA sequences of known *Malassezia* species. The primer sequences were forward 5'-TAACAAGGATTCCCCTAGTA, and reverse 5'-ATTACGCCAGCATCCTAAG. PCR amplification was carried out in a final volume of 50 µl. Each reaction contained 1 µl DNA template, 0.5 µM of each primer, 0.20 mM of each deoxynucleoside triphosphate (dNTPs), 5 µl of 10 × PCR buffer, and 1.25 U of *Taq* polymerase. An initial denaturation step at 94°C for 5 min was followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min, with a final extension step of 72°C for 7 min.

Restriction endonuclease digestions

*Cfo*I and *Bts*CI restriction endonucleases were selected to obtain optimal species-specific patterns as described (13). Each digestion was carried out in a final volume of 30 µl. Each *CfoI* reaction contained 15 µl of PCR reaction products, 1 µl of restriction enzyme, 0.2 µl of acetylated bovine serum albumin (BSA), 3 µl of RE 10 × buffer, and 10.8 µl of autoclaved distilled water. Acetylated BSA and RE 10 × buffer were added to enhance *Cfo*I activity. Each *Bst*CI reaction contained 15 µl of PCR reaction products, 1 µl of restriction enzyme, 3 µl of 10 × NE buffer, and 11 µl of autoclaved distilled water. To enhance *Bts*CI activity, 10 × NE buffer was added. The *Cfo*I and *Bts*CI reaction mixtures were then incubated at 37°C for 3 h and 50°C for 1 h, respectively. PCR products and products of restriction endonuclease digestion were visualized by 1.5% (w/v) and 2% (w/v) agarose gel electrophoresis in TBE buffer, respectively, stained with ethidium bromide, and photographed under UV transillumination.

Direct sequencing

After RFLP analysis, 26 PCR reaction products were direct-sequenced using an ABI PRISM™ Bigdye™ Terminator Cycle Sequencing Ready Reaction Kit V.3.1 (PE Applied Biosystem, USA) to confirm the accuracy of RFLP analysis. Randomly selected *M. furfur* (n = 24), *M. pachydermatis* (n = 1) and *M. obtusa* (n = 1) samples were sequenced and confirmed.

Analysis of standard strain

Standard strains of *M. furfur* (KCTC 7546), *M. pachydermatis* (KCTC 17008) and *M. obtusa* (KCTC 7847) were analyzed as experimental controls. Each freeze-dried yeast strain was diluted in 1 ml of Mueller-Hinton broth, and the diluted

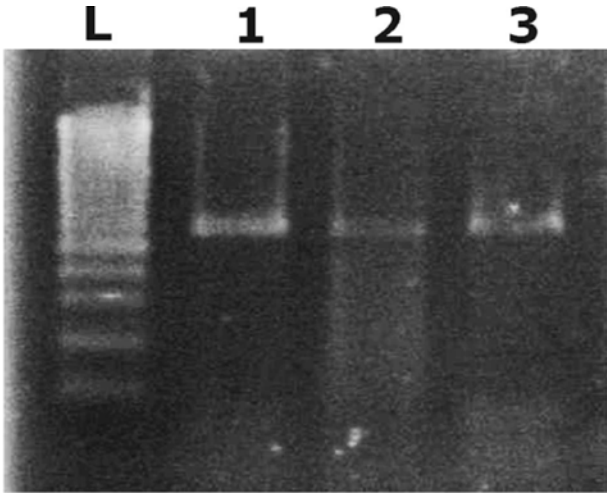

Fig 1. The electrophoretic pattern of PCR products of the standard strains. Etidium bromide stained 2% agarose showing the 26S ribosomal DNA PCR products (approximately 580 bp). L: 100 bp ladder, Lane 1: *M. furfur,* Lane 2: *M. pachydermatis*, Lane 3: *M. obtusa*.

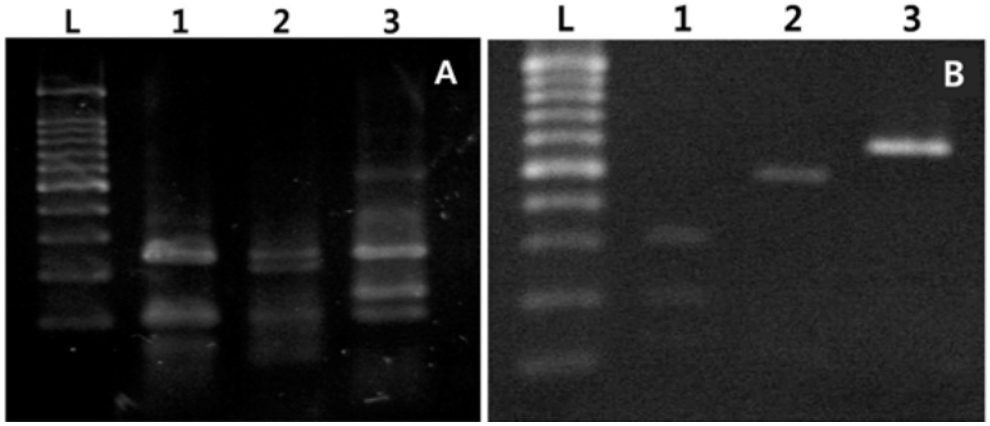

Fig 2. The electrophoretic pattern of restriction fragments of standard strains. (A) The electrophoretic patterns of standard strains digested by *Cfo*I. L: 100 bp ladder, Lane 1: *M. furfur,* Lane 2: *M. obtusa*, Lane 3: *M. pachydermstis*. (B) The electrophoretic patterns of standard strains digested by *Bts*CI. L: 100 bp ladder, Lane 1: *M. furfur,* Lane 2: *M. pachydermatis*, Lane 3: *M. obtusa*.

sample was cultured on modified Dixon medium at 30°C for 7 days. The procedures used for DNA extraction and PCR-RFLP analysis were the same as those used for clinical samples.

Results

Analysis of standard strains by PCR-RFLP

Results from PCR and RFLP analysis of standard strains are shown in Figs 1 and 2. PCR amplification of 26S ribosomal DNA of each standard strain showed a single PCR product of approximately 580 bp. *Cfo*I digestion of PCR amplified products for *M. furfur* showed two different sized fragments (approximately 250 bp and 150 bp); for *M. pachydermatis,* three different sized fragments (approximately 250 bp, 150 bp and 100 bp); and for *M. obtusa*, two different sized fragment (approximately 250 bp and 100 bp). *Bts*CI digestion of PCR amplified products for *M. furfur* showed two different sized fragments (approximately 350 bp and 180 bp); for *M. pachydermatis,* one fragment approximately 480 bp; and for *M. obtusa,* one fragment approximately 550 bp.

Analysis of Clinical samples by PCR-RFLP and direct sequencing

Fifty out of thirty-one *Malassezia* infected patients were investigated by PCR-RFLP. Forty-three samples were extracted from the external ear canal, and seven samples were extracted from the skin surface. The electrophoretic patterns of PCR amplified products and RFLP digestion fragments of clinical samples are shown in the Figs 3 and 4, respectively. After PCR-RFLP, species differentiation of clinical samples was performed using the restriction patterns of the standard strains as a comparison. Table 1 shows the species distribution of isolated clinical samples. *M. furfur* was the most common pathogen (86%). *M. pachydermatis* (4%) and *M. obtusa* (10%) were also identified. All samples from the skin surface were determined to be *M. furfur*. Mixed infections, with several *Malassezia* species, were not found. Direct sequencing of 26 PCR reaction products demonstrated that the RFLP analyses were correct.

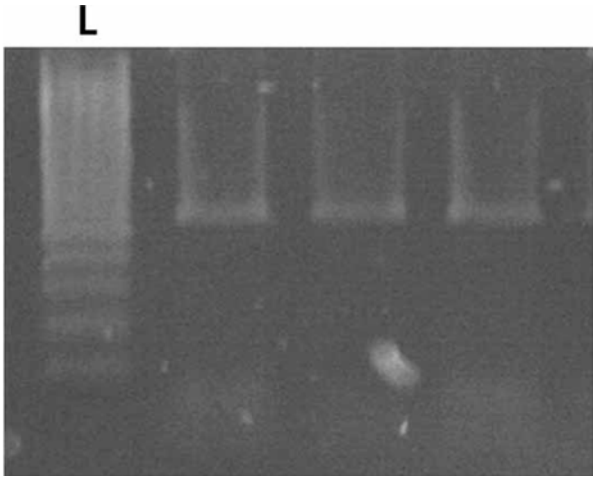

Fig 3. The electrophoretic pattern of clinical samples. Etidium bromide stained 2% agarose showed the 580bp sized bands approximately.

Discussion

In this study, *M. furfur* was identified as a primary pathogen (86%) in infected patients. Only 5 samples were identified as *M. obtusa,* and only 2 samples were identified as *M. pachydermatis*. There were no infections of mixed species. Over the last many years, *M. pachydermatis* has been reported as a main pathogen worldwide in dogs with dermatitis or otitis (2,17). In Spain, the possibility of otitis caused by lipid-dependent species has been confirmed, however such infections represent only a small portion (4.5%) of total *Malassezia*

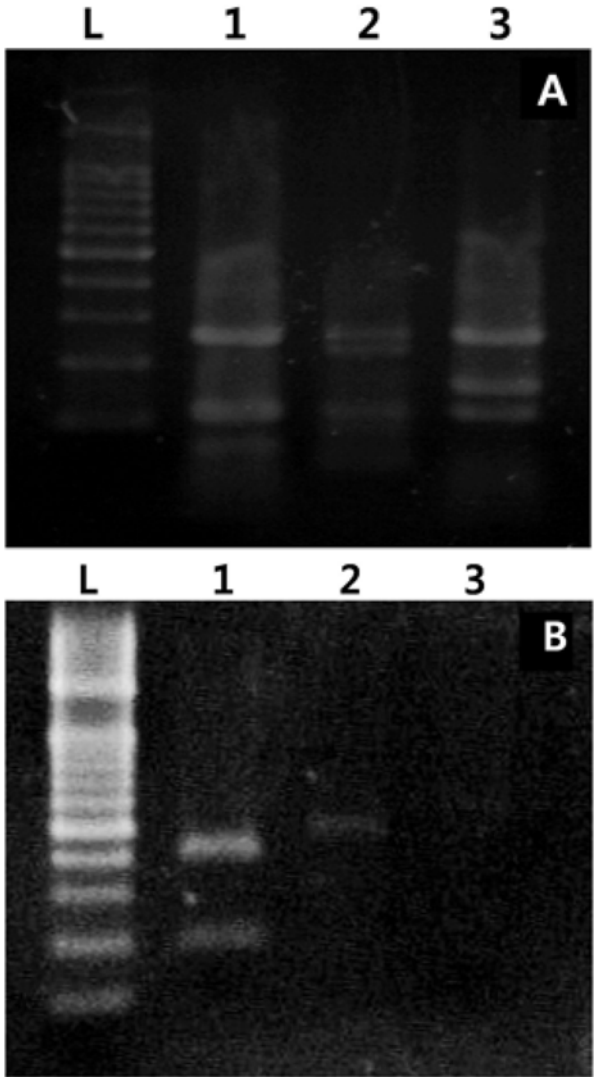

Fig 4. The electrophoretic pattern of restriction fragments of clinical samples. (A) The electrophoretic patterns of clinical samples digested by *Cfo*I (B) the electrophoretic patterns of clinical samples digested by *Bts*CI. L: 100bp ladder, Lane 1: *M. furfur,* Lane 2: *M. pachydermatis*, Lane 3: *M. obtusa*.

infections (6). A study from Italy showed that *M. pachydermatis* exists as a normal microbial flora of skin, but in the same patients lipid-dependent species were increased when infection occurred (14). In their study, the infection by *M. furfur* was attributed to causing 48.6% of the infections in dermatologic patients, including cases of mixed infection; however, *M. furfur* was not main pathogen as well. In this study, samples were collected in Korea, and it is not know whether selected patients displayed lipid-dependent *Malassesia* species as a normal. However, we confirmed that infections by lipid-dependent *Malassesia* species accounted for 96% of total infections. This result is different than that previously reported in other studies.

Several predisposing factors contribute to *Malassezia* infection. Increased humidity and the subsequent overproduction of sebums are probably critical, especially in cases of overgrowth of lipid-dependent species, including *M. furfur* (8,11, 15-17). In this study, 74% of *M. furfur* infections showed no distinct cause for overgrowth of *Malassezia* spp., except alterations of the cutaneous environment. The summer season in the Republic of Korea has an excessively hot and humid climate. It is presumed that this humidity leads to excessive sebum production, and subsequent increases in sebum leads to overgrowth and infections of lipid-dependent species. However, lipid-dependent species were also identified in infections secondary to other diseases that are not associated with an overproduction of sebums. Thus, investigation of the microbial flora should be performed before and after treatment of infected patients, in order to establish the normal flora of *Malassezia* spp. in Korea.

In human medicine, the initiation and aggravation of several inflammatory skin diseases associated with *Malassezia* species were divided into at least two groups. The first group includes pityriasis versicolor and folliculitis. The growth of *Malassezia* directly triggers the development of cutaneous lesions in these diseases. The second group includes atopic dermatitis, seborrheic dermatitis, and psoriasis, which exhibit cutaneous lesions already developed by other mechanisms and are aggravated by the growth of *Malassezia* species (10-12). In veterinary medicine, however, *Malassezia* infections have been suggested as a secondary event, and two mechanisms for triggering overgrowth of yeast have been suggested: alterations in host defense mechanisms, and changes in the cutaneous microenvironment (4). Currently the distribution of *Malassezia* species in these infections does not indicate a difference in predisposing factors.

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개에서 조사된 높은 지방 친화성 *Malassezia* 감염율

한재익 · 조상희 · 나기정[1]

충북대학교 동물의료센터

요 약 : *Malassezia* 종은 온혈 척추동물의 정상 미생물총을 구성하며, 개에서는 비지방친화성인 *M. pachydermatis*가 주로 감염을 유발하는 것으로 알려져 있다. 본 연구는 습도가 높은 한국의 여름 동안 개에 감염을 일으킨 말라세지아 종의 분포를 조사하기 위해 실시되었다. 2006년 6월부터 10월까지 총 50개의 샘플을 채취하여 배양한 후 PCR-RFLP와 direct sequencing을 통해 분석하였다. 조사 결과 지방친화성 종인 *M. furfur*와 *M. obtusa*가 전체 96%를 차지하였으며, *M. pachydermatis*는 4%만을 구성하였다. 이 결과는 기후에 따라 감염을 일으키는 *Malassezia* 종에 차이가 있을 수 있음을 보여준다.

주요어 : 지방친화성 종, *Malasseia* 감염, 개.