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IgG Humoral Immune Response to Extract Proteins of *Malassezia Pachydermatis* Isolated from a Dog with Atopic Dermatitis (Ad)

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Abstract : Malassezia pachydermatis (M. pachydermatis) is a component of the normal cutaneous flora of the dog and atopic dermatitis (AD) is one of the most common diseases associated with Malassezia overgrowth in dogs. The purpose of this study was to investigate the humoral response (IgG) to extracts of M. pachydermatis of in a dog with AD. We used Western immunoblotting to identify allergens of M. pachydermatis. Gel electrophoresis of extracts proteins and immunoblotting of sera samples in both an atopic dog and a non-atopic dog were compared. Proteins of 18, 21, 26, 32, 34, 38, 40, 42, 46, 58, 64, 75, 85, and 120 kDa were observed in a serum of atopic dog. However, when serum of a non-atopic dog was used, protein bands were not identified except binding in 50 kDa protein. The results of this study indicate that atopic dogs with M. pachydermatis dermatitis may induce IgG response and also suggest that humoral response to M. pachydermatis could be important in pathogenesis of AD in dogs. However, further studies are required to identify roles of humoral response to M. pachydermatis in canine AD.

Key words **:** atopic dermatitis, dog, IgG, humoral response, *Malassezia pachydermatis*.

Introduction

Malassezia pachydermatis is commensal yeast from healthy canine skin and mucosa that, under certain circumstance, shows exacerbated development leading to subsequent clinical disease (2). AD is one of the most common diseases associated with *Malassezia pachydermatis* overgrowth in dogs.

Previous studies have shown that levels of *Malassezia*-specific IgG and IgE have elevated in atopic dogs (7, 23). Specific allergens of *Malassezia furfur* have been sequenced and expressed as recombinant proteins in human patient with AD (17, 24, 25, 27, 31). In addition, a number of IgE-binding components in the range of 9-100 kDa have been reported in dogs (15, 19, 32). Likewise, immunoglobulin responses to *Malassezia pachydermatis* and allergenic proteins of it have been studied in atopic dogs (1, 3, 7, 8). Most of the research into the role of antibodies in both human and canine AD has concentrated on immunoglobulin E. However, in experimental animal models, the primary immune responses to allergens delivered to the body are regulated by T-helper 1 cells and T-helper 2 cells which are sensitized by IgG subgroups (13). Therefore to identify correct immune responses to *Malassezia* in canine atopy, it is necessary to investigate IgG reactivity.

The purpose of this study was to identify the humoral immune response (IgG) to extract proteins of *M. pachydermatis* in a dog with AD.

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Materials and Methods

Sera samples

Serum sample was collected from an atopic dog with *Malassezia* dermatitis infection (Fig 1.). Control serum was obtained from clinically normal, 2-month-old, female Cocker Spaniel dog. Serum of each was stored at –20°C until used.

Diagnosis of AD

This dog had clinical signs consistent with the criteria of Willemse (30). Other causes of pruritic skin disease were excluded. Intradermal skin test (IDST) was performed with 40 allergens using 0.05 mL of each allergen extract on the lateral flank after the dog was sedated with medetomidine (20 ug/kg, Dormitor®, Pfizer Animal Health, USA) intravenously and was clipped. Histamine (1/100,000 w/v) and diluents consisting of buffered saline also injected as positive and negative controls, respectively. Test sites were subjectively assessed after 15 min and scored from 0 to +4 by comparison with the controls. Reactions ≥ 2 were considered positive (29).

Skin biopsy was performed, fixed in formalin, and processed to paraffin wax for histological assessment. Sections (5 μm) were cut, routinely processed and stained with Hematoxylin and eosin. An 8-week course of diet trial with commercially available hypoallergenic food (ULTRA z/d®, Hill's, Pet Nutrition, Topeka, KS, USA) was fed to rule out food allergy based on a report described prescribed previously (7). No anti-inflammatory medication was administrated at least 3 weeks prior to examination (7). *M. pachy-dermatis infec-*

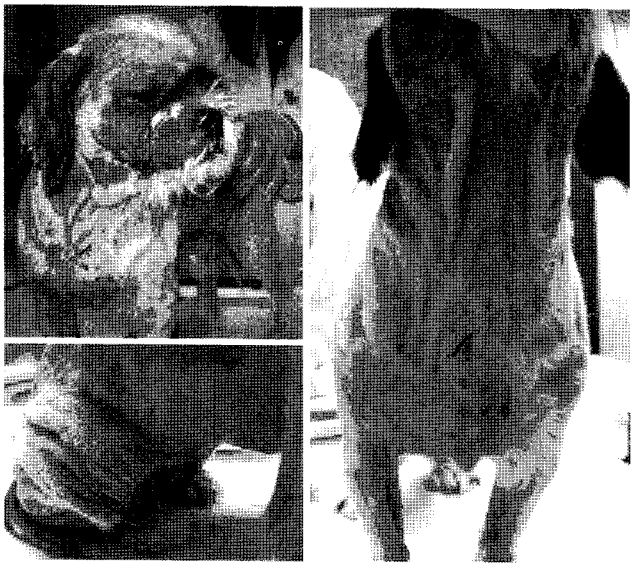

Fig 1. An atopic dog with severe generalized M. pachydermatis dermatitis. Generalized erythema, scale, lichenification, and alopecia were noted.

tion was diagnosed by microscopic observation of Diff-Quik® (Sysmex, Japan) stained tape strips according to the reports described earlier (4, 5, 19). Samples were obtained from the groin, axilla and interdigital web and *Malassezia* overgrowth was characterized as an average of 5 or more *Malassezia* organism per 400 x field (20, 28).

Culture of *M. pachydermatis*

The sample was cultured on Sabouraud dextrose agar (BD, France) containing chloramphenicol (20 mg/ml, Helocetin®, Chong Gun Dang Pharm, Korea) for 48 h at 37°C, and the colonies were identified as *M. pachydermatis* by microscopic examination. An isolate of *M. pachydermatis* was obtained from the skin of the atopic dog. The colonies were then subcultured onto large number of plates in order to obtain enough organisms for subsequent studies.

Extraction of *M. pachydermatis* proteins

Malassezia colonies were carefully harvested and suspended in phosphate-buffered saline (PBS) (pH 7.4) for a washing procedure that consisted of 3 cycles of centrifugation at 500 g for 5 min followed by removal of the supernatant and resuspension in PBS. After the last washing cycle, the cells were resuspended in extraction buffer (pH 7.4) containing 125 mM NH_4HCO_3 (Sigma, USA) and protease inhibitors (20 mM ε-aminocaproic acid, Sigma, USA; 5 mM ethylenediaminetetra-acetic acid, Sigma, USA; and 1 mM phenylmethylsulphonyl fluoride, Sigma, USA) based on a report described previously (15, 22). The *Malassezia* colonies in the extraction buffer were then mixed vigorously with an equal volume of glass beads (0.4 mm, Sigma, USA) on a vortex mixer for 10 min to mechanically disrupt the cell membranes. After extraction, the cell suspensions were stored at 4°C overnight, centrifuged at 6000 g for 5 min and the supernatants were collected. The concentration of protein obtained was measured with protein A 280 method according to a previous report (7).

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis was performed according to the method of Laemmli (16) using a mini-protean 3 system (Bio-Rad, USA) in a discontinuous buffer system containing 0.025 M Tris (BIO-RAD, USA), 0.192 M Glycine (BIO-RAD, USA) and 0.1% sodium dodecyl sulphate (pH 8.3, BIO-RAD, USA). The gel comprised 10% separating polyacrylamide gel and 4% stacking gel. The extracts (20 μg) were diluted 2:1 with reducing sample buffer containing 5% β-Mercaptoethanol, (BIO-RAD, USA) and heated at 95°C for 5 min. The extract was then added to one well across the top of the gel and one well was loaded with molecular markers (Bio-Rad, USA). The electrophoresis was run at 130 V for 60 min.

Western immunoblotting

The separated proteins and molecular weight standards were transferred from the gel to a nitrocellulose membrane (BIO-RAD, USA) in an Electrophoretic Transfer Cell (Bio-Rad Trans-Blot® SD Semi-Dry, USA). according to the manufacturer's instructions. The transfer buffer contained 48 mM Tris (Sigma, UK), 39 mM Glycine (BIO-RAD, USA), 0.0375% SDS (BIO-RAD, USA) and 20% methanol (Duksan chemical, Korea), pH 8.9. The transfer was run at 15 V per minigel for 20 min.

For immunoblotting, the sera samples were analyzed using the following conditions. The nitrocellulose membrane was removed and washed in Tris-buffered saline pH 7.5 (TBS) for 30 minutes with gentle rocking. In addition, the prestained molecular weight markers (Bio-Rad, USA) were included. The residual binding sites on the nitrocellulose membrane were blocked by incubation in TBS containing 1% skimmed milk for 30 minutes at room temperature with gentle rocking. The membrane was washed with PBS containing 0.05% Tween 20 (Sigma, USA) (PBST) for 30 min (3 × 10 minutes rinses). The membrane was incubated with serum at 1/100 in PBST for 2 hours at room temperature. The membrane were washed with PBST for 30 min (3 x 10 minutes rinses), and then incubated for 2 hrs at room temperature with hydrogen peroxide (HRP)-conjugated rabbit anti-dog IgG (Sigma, USA) at 1/2000 in PBST. After washing with PBST for 30 min (3 × 10 minute rinses), the membrane were developed by incubating them with substrate solution containing 4-chloro-1-naphthol (CN, Bio-Rad, USA) (30 mg/ml in methanol) for 25 min. The molecular weight of the visualized bands was calculated by their relative mobility from the regression line of the $\log_{10}$ of the molecular weight of the standard proteins plotted against their relative mobility.

Results

Intradermal skin test (IDST)

The atopic dog showed positive reactions to five allergens in IDST (Fig 2). The causative allergens were *Rhizopus* mix, House dust mixture, Silk, House dust mites (*Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*) (Table 1).

Histopathology

Histopathology of *Malassezia* dermatitis with AD is described in Figure 3. Marked epidermal spongiosis and parakeratotic hyperplasia were revealed in the epidermis. Some apoptotic keratinocytes were shown. Dermal edema and diffuse dermatitis were also noted.

SDS-PAGE and Western immunoblotting

In this study, M. pachydermatis protein extracts were electrophoresed by SDS-PAGE on a 10% separating polyacrylamide gel and stained with Coomassie Brilliant Blue R-250 (Fig 4).

The immune reactivity of *M. pachydermatis* proteins identified by serum from a dog with AD was presented in Figure 5. 16 proteins of 18, 21, 26, 32, 34, 38, 40, 42, 46, 50, 58, 64, 75, 85, 120 kDa were detected in the atopic dog. Protein band of 50 kDa was detected in the control serum.

Discussion

Atopic symptoms and the presence of positive skin prick tests and circulating IgE antibodies to allergens were associated with high levels of IgG subclass antibody responses to allergens in human literatures described previously (14, 26). Serum total IgG concentrations or allergen specific IgG levels have been also shown to be increased by infections and AD in dogs (10, 12, 21, 23). These findings of earlier studies suggested that immunoreactivity of *Malassezia* in AD might be associated with IgG levels in dogs.

The present study investigated the humoral immune response (IgG) to *M. pachydermatis* isolated from an atopic dog by the immunoblotting. Protein bands of 18, 21, 26, 32, 34, 38, 40, 42, 46, 58, 64, 75, 85, and 120 kDa were observed in the atopic dog and not in non-atopic dogs, suggesting that response to these proteins may be related to the development of the disease. According to previous studies (3, 7), IgG responses to *M. pachydermatis* in atopic dogs were significantly higher than healthy dogs. Positive correlation between the number of bands identified and the optical density values of *M. pachydermatis*-specific IgG antibodies in each sample were previously determined (1). Higher reactiv-

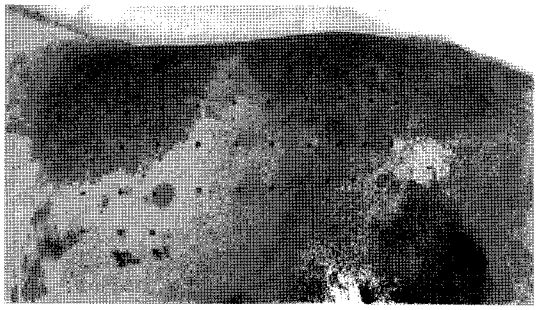

Table 1. Result of IDST in an atopic dog

Allergen groups	Allergens	Reactivity
Pollen	Bermuda Grass	
Weeds	Cocklebur	
	Goldenrod	
	Lamb's Quarter	
	Pigweed, Rough/Redroot	
	Plantain, English	
	Dandelion	
	Sage Mix	
Tree and Shrubs	Ragweed Mix	
	Alder, White	
	Hazelnut American	
	Birch Mix	
	Pine Mix	
	11 Tree Mix	
Molds	*Candida albicans*	
	Cephalosporium acremonium	
	Cephalothecium roseum	
	Fusarium moniliforme	
	Fusarium solani	
	Trichophyton mentagrophytes	
	Trichophyton rubrum	
	Aspergillus Mix	
	Penicillium Mix	
	Mucor Mix	
	Rhizopus Mix	Positive
Smut	Grass Smut Mix	
	Grain Smut Mix	
House dust	Dust, House Mixture	Positive
Epidermal and inhalants	Cat epithelia	
	Cotton seed	
	Kapok seed	
	Pyrethrum	
	Silk	Positive
	Mixed Feathers	
House dust mites (HDMs)	*Dermatophagoides farinae*	Positive
	Dermatophagoides pteronyssinus	Positive
Insects	Flea	
	Mosquito	
	2 Cockroach Mix	

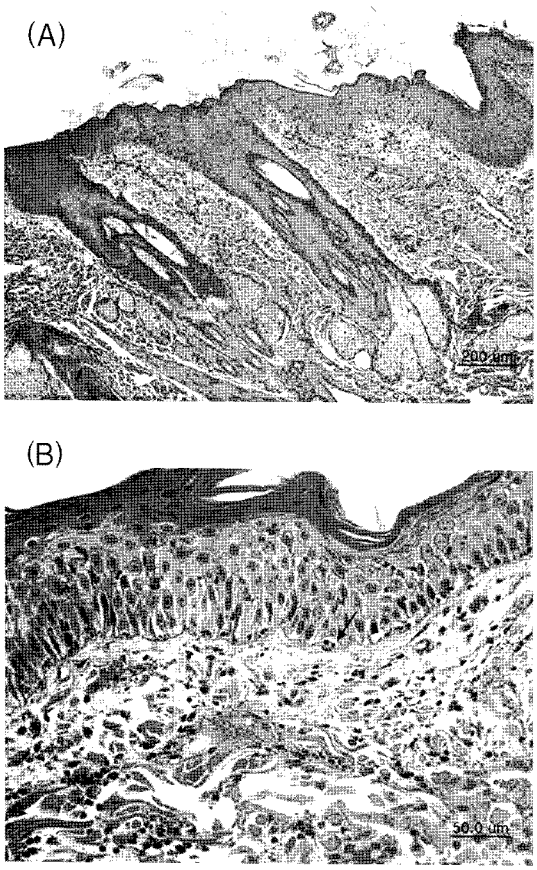

Fig 3. Histopathology of canine *M. pachydermatis* dermatitis with AD. Hematoxylin and eosin stain. (A): Epidermal hyperplasia and mild hyperkeratosis. Hyperplasia of sebaceous glands is noted around follicles (× 100). (B): The epidermis shows marked spongiosis and parakeratotic hyperplasia. Some apoptosis of keratinocytes were shown (thin arrow). Dermal edema and diffuse dermatitis were noted (× 400).

ity of *M. pachydermatis*-specific IgG antibodies in the atopic dog than the non-atopic dog was found in this study. This finding indicates that the proliferation of the yeast and AD might be associated with an enhanced humoral immune response.

Previous studies (3, 6, 9) showed identification of major allergens of *M. pachydermatis* in aspect of number of bands and molecular weight. The results of the present study are very similar to those reported by one study which described IgG responses to *M. pachydermatis* among healthy and atopic dogs (7). It also reported that all dogs included in the study with different diseases showed commonly three antigens of 42, 45 and 82 kDa. Antigens of 25, 29, 61, 82 and 90 kDa were also found described in atopic dogs. However, according to a report described previously (9), the antigenic composition of *M. pachydermatis* extracts could depend on the duration of yeast incubation and the phase of yeast growth in culture.

This study demonstrates that IgG-mediated humoral immune response was elicited following *M. pachydermatis*

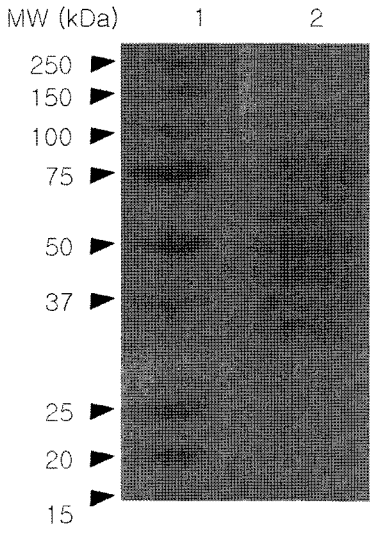

Fig 4. Coomassie Brilliant Blue stained *M. pachydermatis* protein extracts on 10% separating polyacrylamide gel. Lane 1: molecular weight marker; lane 2: *M. pachydermatis* protein extract.

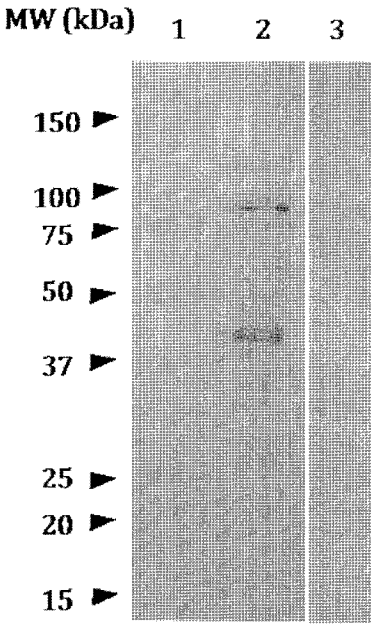

Fig 5. IgG-binding proteins in extracts of *M. pachydermatis* detected by Western immunoblotting with serum from an atopic dog and non-atopic dogs (control). Lane 1: molecular weight marker; lane 2: IgG binding proteins in an atopic dog serum; lane 3: IgG binding proteins in non-atopic dog serum.

infection of a dog with AD. And that humoral response other than IgE-mediated could be crucial in the pathogenesis of canine AD with *M. pachydermatis* infection.

The limitation of this study is that a limited number of dogs were used as a preliminary study for immune response. Therefore, further work with a number of sera is required on the extract role played by IgG response to *M. pachydermatis* in AD.

Conclusion

In conclusion, this study suggests that *M. pachydermatis* in AD could cause significant humoral immune response to a select number of proteins on *M. pachydermatis*. Further studies are required to identify the exact role of IgG in AD with *M. pachydermatis* infection.

Acknowledgement

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아토피 견에서 분리한 *M. pachydermatis* 추출 단백질에 대한 IgG 체액성 면역 반응의 연구

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요 약 : *M. pachydermatis*는 개에서의 피부 정상 균 종으로서, 과잉 증식되는 가장 흔한 질병 중의 하나가 아토피성 피부염이다. 이 연구의 목적은 개의 아토피성 피부염에서 *M. pachydermatis* 추출 단백질에 대한 IgG를 이용한 체액성 면역 반응을 알아보는 것이다. *M. pachydermatis*의 전기 영동과 개의 혈청을 이용한 Western immunoblotting을 이용해 *M. pachydermatis*의 알러젠을 밝히고 아토피 견과 비 아토피 견에서의 각각의 면역 반응을 비교하였다. 결과는 아토피 견의 혈청에서 18, 21, 26, 32, 34, 38, 40, 42, 46, 58, 64, 75, 85, 그리고 120 kDa의 단백질의 반응이 발견된 것에 반해, 비 아토피 견에서는 50 kD을 제외한 다른 단백질의 반응은 발견되지 않았다. 이 연구의 결과로 *M. pachydermatis* 피부염을 지닌 개의 아토피성 피부염은 IgG의 체액성 면역 반응을 유발하고, 이 면역 반응은 개의 아토피성 피부염의 발병 기전에 중요한 역할을 한다는 것을 알 수 있다. 그러나 개의 아토피성 피부염에서의 *M. pachydermatis*에 대한 체액성 면역 반응의 역할을 밝히기 위해서는 더 많은 연구가 필요하다.

주요어 : 아토피성 피부염, 개, IgG, 체액성 면역 반응, *Malassezia pachydermatis*