

The immunoglobulin G response to *Malassezia pachydermatis* extracts in atopic and non-atopic dogs

Ha J. Kim, Eun T. Kim, Chae Y. Lim, Chul Park, Byeong T. Kang, Ju W. Kim, Jong H. Yoo, Hee M. Park

Abstract — IgG immunoreactivity to *Malassezia pachydermatis* was compared in atopic and non-atopic dogs. *Malassezia pachydermatis* proteins with a molecular weight of 98 kDa were recognized at a significantly higher frequency in the sera of atopic dogs. Most of the atopic dogs with *Malassezia* dermatitis had a greater IgG response than did normal dogs.

Résumé — La réponse en immunoglobulines G à des extraits de *Malassezia pachydermatis* chez les chiens atopiques et non atopiques. L'immunoréactivité des IgG envers *Malassezia pachydermatis* a été comparée chez des chiens atopiques et non atopiques. Des protéines de *Malassezia pachydermatis* avec un poids moléculaire de 98 kDa ont été reconnues à une fréquence significativement supérieure dans le sérum des chiens atopiques. La plupart des chiens atopiques avec une dermatite à *Malassezia* présentaient une réponse en IgG supérieure à celle des chiens normaux.

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Introduction

Malassezia pachydermatis is frequently isolated from the skin and mucosal sites of healthy dogs, but some dogs with dermatitis have remarkably increased populations of the yeasts, suggesting that the organism is an opportunistic pathogen (1). Previous studies have suggested that *Malassezia* species induce both humoral and cell-mediated immune responses in humans and dogs (2,3). Elevated *Malassezia*-specific serum IgG and IgE have been identified in atopic dogs (4). Atopic dogs also have elevated serum IgE titers to *M. pachydermatis*, which frequently show immunoreactivity to antigens of 45, 52, 56, and 65 kDa (5), and dogs with *M. pachydermatis* have a greater IgG response to a clinically important 25 kDa antigen when compared with control animals (6). Furthermore, anti-*M. pachydermatis* IgG immunoreactivity towards proteins with molecular weights of 219, 110, 71, and 42 kDa has been observed in affected dogs (7). The results of many previous studies designed to identify antigenic proteins of *M. pachydermatis*

and the role of immunoglobulins have been so variable that the pathogenesis of *M. pachydermatis* is still unclear.

The purpose of this study was to compare the IgG immune responses to *M. pachydermatis* in atopic and non-atopic dogs and to identify antigenic proteins of the yeast.

Materials and methods

Serum samples

Serum samples were collected from 14 atopic dogs and 14 non-atopic dogs presented to the Konkuk University Veterinary Teaching Hospital.

Identification of dogs with atopic dermatitis

Identification of atopic dermatitis (AD) was based on a combination of consistent history and clinical signs, and exclusion of other causes of pruritic skin diseases (8). We performed skin scraping to exclude ectoparasites. In addition, antiparasitic control included monthly topical application of Fipronil (Frontline; Merial, Lyon, France), systemic Ivermectin (Virbamec; Virbac Laboratorios, Guadalajara, Mexico), 0.5 mg/kg, SC, weekly for 1 mo, and bathing with amitraz (Greentix; Green Cross Veterinary Products, Young-In, Korea) once a week for 2 wk.

The atopic dogs were fed an 8-week trial diet with a commercial hypoallergenic food to rule out food allergy. No anti-inflammatory medication was given for at least 3 wk prior to examination (5). Four of the dogs were clipped, sedated (medetomidine, Pfizer Animal Health, New York, New York, USA), 20 µg/kg, IV, and intradermal skin tests were performed with 40 allergens (Greer laboratories, Lenoir, North Carolina, USA) using 0.05 mL of each allergen extract on the lateral flank. Histamine (1:100 000 w/v) and a buffered-saline diluent were also injected as positive and negative controls, respectively. Test sites were assessed after 15 min and scored from 0 to + 4 by

Department of Veterinary Internal Medicine, College of Veterinary Medicine, Konkuk University, #1 Hwayang-dong Kwang-jin-gu, Seoul 143–70, South Korea (Ha Kim, Eun Kim, Lim, Kang, Ju Kim, Yoo, Hee Park); Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California 95616, USA (Chul Park).

Ha J. Kim and Eun T. Kim contributed equally to this work.

Address all correspondence to Dr. Hee M. Park; e-mail: parkhee@konkuk.ac.kr

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comparison with the controls. Reactions ≥ 2 were considered to be positive (9).

Firm cytological criteria for *Malassezia* overgrowth have not been established, and there are important breed and site differences in yeast numbers in healthy dogs (10). *Malassezia* overgrowth was diagnosed by microscopic observation of Diff-Quik (Sysmex, Kobe, Japan) stained tape strips. Samples were obtained from the groin, axilla, and interdigital areas. For this study, *Malassezia* overgrowth was defined as an average of 5 or more *Malassezia* organisms per 400 $\times$ field (5,10).

Non-atopic dogs had no history or clinical signs of skin disease and had no lesions upon dermatological examination. Most of the non-atopic dogs had been presented for other diseases.

Culture of *Malassezia pachydermatis*

An isolate of *M. pachydermatis* was obtained from the skin of an atopic dog with *Malassezia* overgrowth. The sample was cultured for 48 h at 37°C on Sabouraud dextrose agar (Becton Dickinson, Le Pont de Claix-Cedex, France) containing 20 mg/mL chloramphenicol (Helocetin; Chong Gun Dang Pharm, Korea), and the colonies were identified as *M. pachydermatis* by microscopic examination. The colonies were subcultured onto a large number of plates to obtain an adequate number of organisms for the subsequent studies (5).

Extraction of *Malassezia pachydermatis* proteins

Malassezia colonies were harvested, suspended in phosphate-buffered saline (PBS) (pH 7.4), and washed by 3 cycles of centrifugation at 500 $\times g$ for 5 min followed by removal of the supernatant and resuspension of the pellet in PBS. After the last washing cycle, the cells were resuspended in an extraction buffer (pH 7.4) containing 125 mM NH_4HCO_3 (Sigma, St. Louis, Missouri, USA) and protease inhibitors (20 mM ϵ -aminocaproic acid, 5 mM ethylenediaminetetra-acetic acid, and 1 mM phenylmethylsulfonyl fluoride; Sigma) (11,12). The *Malassezia* colonies in the extraction buffer were mixed vigorously on a vortex mixer for 10 min with an equal volume of glass beads (0.4 mm; Sigma) to mechanically disrupt the cell membranes. After extraction, the cell suspensions were stored at 4°C overnight, centrifuged at 6000 $\times g$ for 5 min and the supernatants were collected. The amount of protein obtained was measured by the protein A 280 method (13).

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis was performed according to the method of Laemmli (13) using a mini-protean 3 system (Bio-Rad, USA) in a discontinuous buffer system containing 0.025 M Tris (Bio-Rad), 0.192 M Glycine (Bio-Rad), and 0.1% SDS (Bio-Rad), pH 8.3. The gel was comprised of a 10% separating and a 4% stacking gel. The extracts (400 μg) were diluted 2:1 with reducing sample buffer (containing 5% β -mercaptoethanol, Bio-Rad), heated at 95°C for 5 min and then loaded onto gels next to a lane loaded with molecular markers (Bio-Rad). The electrophoresis was run at 130 V for 60 min and the gel stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

Western immunoblotting

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

The individual serum samples were analyzed under the following conditions. The nitrocellulose membranes were removed and washed in Tris-buffered saline pH 7.5 (TBS) for 30 min with gentle rocking. The residual binding sites were blocked by incubating the membranes in TBS containing 1% skimmed milk for 30 min at room temperature with gentle rocking. The membranes were washed with PBS containing 0.05% Tween 20 (Sigma, USA) (PBST) for 30 min (three, 10-minute rinses), cut into vertical strips (5-mm wide), and then each strip was placed into a small petri dish. A strip was incubated with each serum sample at 1/100 in PBST for 2 h at room temperature. The membranes were washed with PBST for 30 min (three, 10-minute rinses), and then incubated for 2 h at room temperature with Horseradish Peroxidase (HRP)-conjugated rabbit anti-dog IgG (Sigma, USA) at 1:2000 in PBST. After washing with PBST for 30 min (three, 10-minute rinses), the strips were developed for 25 min in a substrate solution containing 4-chloro-1-naphthol (30 mg/mL in methanol). The molecular weights of the visualized bands were calculated by their relative mobility from the regression line of the log₁₀ of the molecular weight of the standard proteins plotted against their relative mobility. The gel was analyzed using image analysis software (ImageJ 1.40g, National Institutes of Health, USA).

Statistical analysis

The bands were classified into negative, weak, and strong reaction based on their intensity. The intensities of zero to half of the maximum intensity, half to the maximum intensity, were defined as weak and strong, respectively. Fisher's exact test was used to assess the differences between the percentages of atopic and non-atopic dogs showing strong IgG binding to individual proteins in the *M. pachydermatis* extracts.

Results

Age, sex, and breed of the study animals and the diseases of the non-atopic dogs are detailed in Table 1. The mean ages of atopic and non-atopic dogs were 6.0 y and 3.4 y, respectively. Of the 28 dogs in the study, 15 were male and 13 were female. Only 1 dog was healthy; the others had various systemic diseases.

The extracts of *M. pachydermatis* were separated in SDS-PAGE. The IgG immunoblots of dog sera from atopic dogs and non-atopic dogs are shown in Figure 1. The IgG binding proteins with molecular weights of 73 (50% of dogs), 74 (50%), 86 (71%), 88 (71%), 98 (50%) kDa were recognized in > 50% of the atopic dogs. Most of the non-atopic dogs showed weak binding to those proteins, and strong binding was detected in 28.5%, 21.4%, 50%, 42.8%, and 7.1%, respectively. The differences between atopic and non-atopic dogs in IgG binding to the main proteins of *M. pachydermatis*, however, were not

Table 1. Age, sex, breed and diagnosis of atopic and non-atopic dogs

Atopic dogs	Age (y)	Sex	Breed	Diagnosis
1	8	CM	King Charles spaniel	AD, Mitral valve insufficiency
2	12	NF	Yorkshire terrier	AD
3	3.5	NF	Shih tzu	AD, <i>M. pachydermatis</i> overgrowth
4	5	IF	Shih tzu	AD, PDH, FA
5	12	IM	Cocker spaniel	AD, <i>M. pachydermatis</i> overgrowth
6	5	CM	Pug	AD, <i>M. pachydermatis</i> overgrowth
7	3.5	CM	Shih tzu	AD, <i>M. pachydermatis</i> overgrowth
8	4.5	NF	Shih tzu	AD, Generalized demodicosis
9	12	CM	Shih tzu	AD
10	5.5	IF	Cocker spaniel	AD, IMHA
11	2	CM	Schnauzer	AD, FA
12	1.5	CM	Pekingese	AD
13	8	CM	Miniature pincher	AD
14	2	IF	Pekingese	AD
Non-atopic dogs	Age (y)	Sex	Breed	Diagnosis
15	9	IF	Yorkshire terrier	Pyometra
16	2 mo	IM	Cocker spaniel	Healthy
17	5	NF	Labrador retriever	Idiopathic epilepsy
18	3	CM	Maltese	IMHA
19	8 mo	IM	Shih tzu	Canine parvoviral infection
20	1	IF	Siberian husky	IMT
21	4	CM	Pomeranian	Intervertebral disc disease
22	5	IM	Cocker spaniel	Chronic hepatitis, cirrhosis
23	7	IM	Yorkshire terrier	Acute pancreatitis
24	2	IM	Mixed	Hydrocephalus, Atlanto-axial instability
25	2	IF	Yorkshire terrier	Tracheal collapse, tracheobronchitis
26	2 mo	IF	Shih tzu	Immune-mediated polyarthritis (type I)
27	4	IF	Akita	Intestinal lymphoma, PLE
28	5	IM	Siberian husky	Medial nerve neuropathy

AD — atopic dermatitis; PDH — pituitary-dependent hyperadrenocorticism; FA — food allergy; IMHA — immune-mediated hemolytic anemia; IMT — immune-mediated thrombocytopenia; PLE — protein losing enteropathy; y — year; mo — month; CM — castrated male; IM — intact male; NF — neutered female; IF — intact female.

statistically significant except for the 98 kDa protein ($P < 0.05$) (Figure 1).

Discussion

Malassezia pachydermatis is a resident organism in the canine stratum corneum. Previous studies (5,6) have shown that both healthy dogs and dogs with *M. pachydermatis*-related skin disease had serum IgG antibodies to the yeast. Cutaneous hypersensitivity, including AD, is a common underlying cause of alterations to the cutaneous microclimate or to the host defense mechanisms allowing *M. pachydermatis* to multiply and cause disease. Atopic dogs, therefore, may have a greater sensitivity to the variety of immune responses induced by *M. pachydermatis*.

Previous studies have reported that atopic dogs, with clinical evidence of *Malassezia* dermatitis and otitis, had significantly higher serum IgG and IgE levels than either healthy dogs or non-atopic dogs (4,5). Another study showed that atopic working dogs had a significantly higher concentration of serum total IgG than non-atopic working dogs (14). These results suggest that immunoreactivity to *M. pachydermatis* in atopic dogs could be associated with an increased concentration of serum IgG or IgE.

In our study, the intensity of IgG binding seen in atopic dogs was higher than that in the non-atopic dogs for most of the identified *M. pachydermatis* proteins, although the differences were statistically significant for only the 98 kDa protein. Our study showed that atopic dogs had a high IgG immunoreactivity

to the *M. pachydermatis* proteins with molecular weights of 73, 74, 86, 88, 98 kDa, suggesting that these specific yeast proteins may be relevant in atopic dogs. These observations do not agree with the results of previous studies in which proteins of different molecular weights were identified (5,6). The differences in immunoreactivity to *M. pachydermatis* between different studies could be related to differences in the phase of yeast growth, individual host responses, strain variability, and the different extraction processes affecting antigen release and stability (15). In addition, the different results could be related to differences in immunoreactivity between IgG and IgE in various studies.

According to a previous report (6), the IgG may act as an opsonin to facilitate phagocytosis of *Malassezia* antigens. This could have a protective function but it could also activate the complement system and exacerbate the underlying inflammation. It has been shown that *Malassezia* is capable of activating both the classical and alternative complement pathways (16).

In our study, an antigen of 98 kDa and of unknown function and identity was recognized by 50% of the atopic dogs and only 7.1% of the non-atopic dogs. Protein purification and N-terminal amino acid sequencing may assist in positively identifying this antigen and the other relevant antigens of *M. pachydermatis* with molecular weights of 73, 74, 86, and 88 kDa.

In conclusion, this study demonstrates that total serum IgG immunoreactivity to *M. pachydermatis* antigens was higher in atopic dogs than non-atopic dogs, particularly with a 98 kDa antigen.

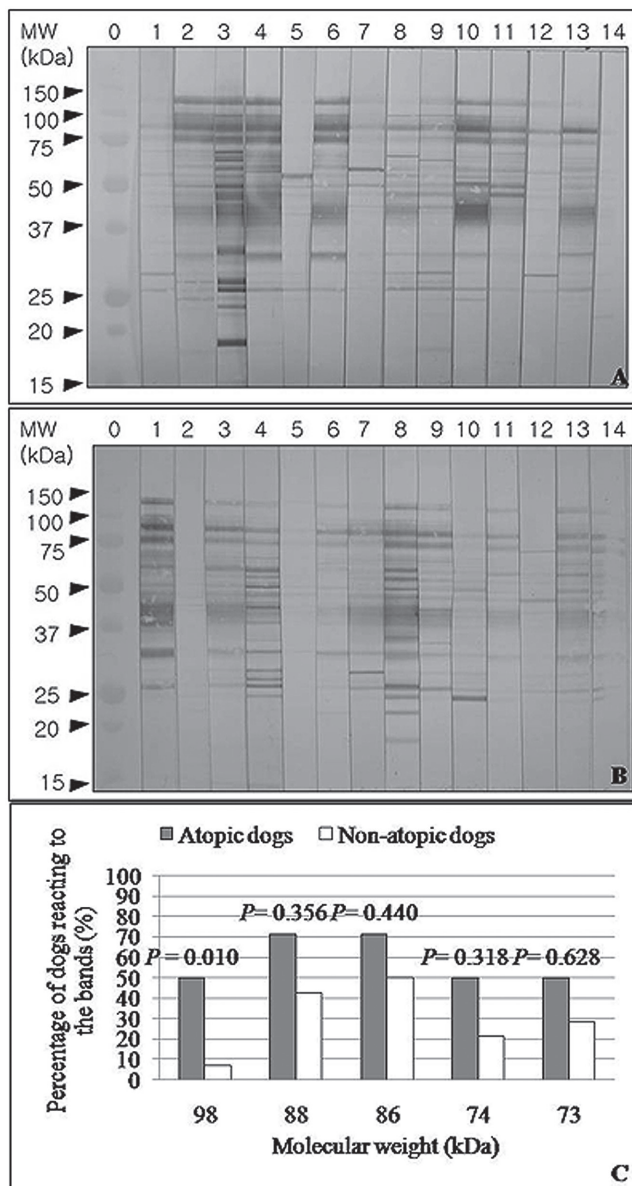


Figure 1. Western immunoblot of the IgG-binding proteins in *M. pachydermatis* extracts detected in sera of atopic (A) and non-atopic dogs (B). Lane 0 – molecular weight markers; Lanes 1–14 – serum samples from atopic and non-atopic dogs; the percentage of atopic and non-atopic dogs showing strong IgG binding to individual proteins in *M. pachydermatis* extracts. x-axis; molecular weights of the detected bands; y-axis; percentage of dogs reacting to the protein of the specified molecular weight (C). The percentages of atopic and non-atopic dogs reacting to the 98 kDa protein were significantly different ($P < 0.05$).

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