

Research paper

IgE sensitivity to *Malassezia pachydermatis* and mite allergens in dogs with atopic dermatitis

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ABSTRACT

In this study, dogs with atopic dermatitis were separated into non-food-induced atopic dermatitis (NFIAD) group (n = 15) and food-induced atopic dermatitis (FIAD) group (n = 37) based on an elimination diet test. IgE reactivity for crude *Malassezia pachydermatis* (*M. pachydermatis*) and house dust mites (HDM) allergen extracts was investigated in the two groups using fluorometric enzyme-linked immunosorbent assay (ELISA) and intradermal skin test (IDST). Nine (60%) of the 15 dogs in NFIAD group and 6 (16%) of the 37 dogs in FIAD group showed specific IgE for *M. pachydermatis* (Mann-Whitney U-test, $P < 0.01$). By immunoblotting analysis, the pooled serum samples from dogs with IgE for *M. pachydermatis* showed IgE reactivity for 50 kDa protein of *M. pachydermatis*. Twelve (80%) of the 15 dogs in NFIAD group and 8 (22%) of the 37 dogs in FIAD group showed specific IgE for HDM (Mann-Whitney U-test, $P < 0.01$). In addition, the dogs in NFIAD group significantly show a positive IDST to *M. pachydermatis* and HDM extracts compared with the dogs in FIAD group. The results suggest that dogs with NFIAD are at increased risk of becoming sensitized to the normal commensal organism *M. pachydermatis* compared with dogs with FIAD, perhaps co-sensitization occurred due to an HDM protease antigen's, Der f 1 and/or Der p 1, proteolytic activity related epidermal skin barrier defects. Treatment to limit skin colonization may thus be especially important in NFIAD.

1. Introduction

Canine atopic dermatitis (CAD) is a common disease in veterinary dermatology (Willemse, 1986; Favrot et al., 2010; Olivry et al., 2010). The clinical signs of CAD include pruritus, otitis externa, and inflammatory, recurrent, and chronic dermatitis (Willemse, 1986). The diagnosis of CAD is based primarily on the atopic dog's signalment (e.g., age of onset, and breed), clinical signs and disease history. Several sets of criteria have been developed for the diagnosis of CAD (Willemse, 1986; Prélaud et al., 1998; Favrot et al., 2010). Based on the most recent Favrot's criteria, CAD can be classified as follows: 1) non-food-induced atopic dermatitis (NFIAD) which is associated with sensitization to environmental allergens; 2) food-induced atopic dermatitis (FIAD) which is associated with sensitization to food allergens with the same signs as CAD; and 3) atopic-like dermatitis (ALD) which is clinical signs of CAD, but no demonstrable allergen-specific IgE (Favrot et al., 2010). Most cases fit into either one of the first two groups (Favrot

et al., 2010). Since NFIAD and FIAD have almost identical clinical signs, the definitive diagnosis can only be made by elimination diet test (Verlinden et al., 2006; Favrot et al., 2010).

Malassezia pachydermatis (*M. pachydermatis*) is commensal yeast that is commonly found on mammalian skin and has been recognized as a very common cause of dermatitis in dogs (Bond et al., 2006). Meason-Smith et al. (2015) has reported *Malassezia* species are the skin-associated microbiome in both healthy and atopic dogs. Previous studies have shown that most dogs harbor commensal *Malassezia* populations, particularly in the ear and at mucosal sites (Nuttall and Halliwell, 2001; Oldenhoff et al., 2014). Morris et al. (1998) has compared atopic dogs with and without *Malassezia* dermatitis based on the results of intradermal skin test (IDST) against *M. pachydermatis* extract and found a significant difference between the two groups. Nuttall and Halliwell (2001) has reported that the serum levels of both anti-*M. pachydermatis* IgG and IgE were higher in atopic dogs than in healthy dogs, regardless of the presence of clinical evidence of *Malassezia* dermatitis and/or

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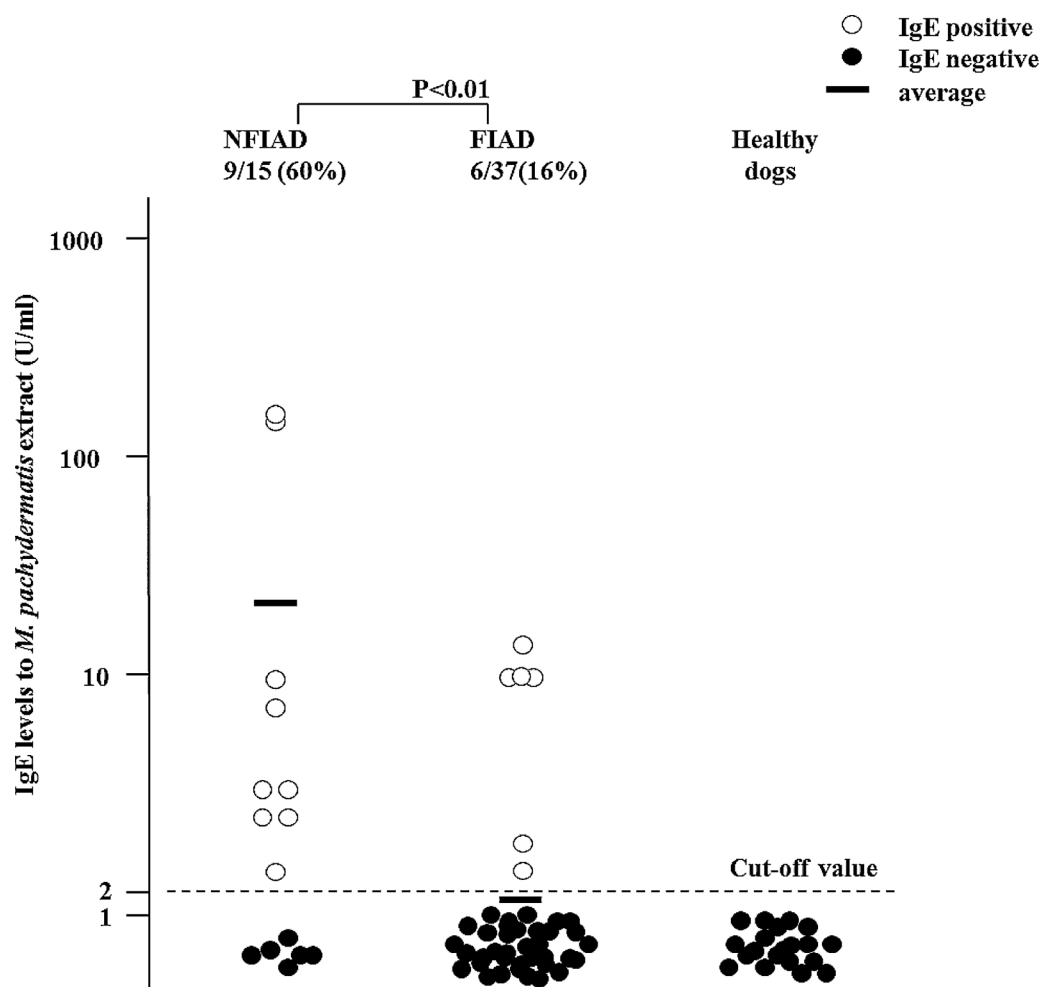


Fig. 1. Specific IgE against *M. pachydermatis* extract in NFIAD and FIAD groups and healthy dogs. The cut-off value was the average $\pm$ 3X standard deviation (SD) of the IgE level of 20 healthy dogs.

otitis externa in the atopic dogs at the time of sampling. Using Prausnitz-Küstner test, Morris and DeBoer (2003) has confirmed the presence of anti-*M. pachydermatis* IgE in dogs with CAD. Oldenhoff et al. (2014) has reported that result of IDST and ELISA against *M. pachydermatis* agreed substantially. Layne and DeBoer (2016) reported that dogs affected with atopic dermatitis had immediate-type hypersensitivity to *Malassezia*.

The purpose of the present study was to examine the allergen-specific hypersensitivity against *M. pachydermatis* in dogs with CAD. Dogs were diagnosed as CAD based on the criteria of Prélard (Prélard et al., 1998), and then classified into NFIAD and FIAD groups according to Favrot's criteria. In the present study, fluorometric ELISA was used to measure specific IgE against *M. pachydermatis* and house dust mites (HDM), *Dermatophagoides farinae* and *D. pteronyssinus*, in the NFIAD and FIAD groups and in healthy dogs (control group). In addition, the NFIAD and FIAD groups underwent IDST using *M. pachydermatis* and HDM extracts.

2. Materials and Methods

2.1. Diagnosis of CAD

This study included dogs that were diagnosed at Fujimura Animal Hospital, Osaka, Japan. After ruling out other skin conditions with clinical signs that can resemble, or overlap with CAD (i.e., flea allergy dermatitis, ectoparasites, bacterial skin infections, and *Malassezia* dermatitis), the dogs were diagnosed with CAD based on Prélard's criteria

(Prélard et al., 1998). Next, the CAD dogs were grouped into two groups: 1) dogs with clinical signs of atopic dermatitis and negative response to elimination diet test (NFIAD group), and 2) dogs with clinical signs of atopic dermatitis and positive response to a 6- to 8-week elimination diet and subsequent allergen specific food challenge (FIAD group) (Favrot et al., 2010). Only dogs with either NFIAD or FIAD were chosen for the study. Dogs with concurrent NFIAD and FIAD or those with ALD, were excluded. Finally, a total of 52 dogs were enrolled in this study; 15 were in NFIAD group and 37 were in FIAD group.

2.2. Mites and *M. pachydermatis* antigens

Crude *M. pachydermatis* extract was purchased from Greer Laboratories (Lenoir, NC, USA). Crude HDM extracts of *D. farinae* and *D. pteronyssinus* were provided by Institute of Tokyo Environmental Allergy (Tokyo, Japan).

2.3. Measurements of IgE to crude extracts of *M. pachydermatis* and HDM allergens using fluorometric ELISA

Specific IgE antibodies to *M. pachydermatis* and HDM (*D. farinae* and *D. pteronyssinus*) allergens were assayed using fluorometric ELISA (Sakaguchi et al., 1997; Shimakura et al., 2016). A microplate (NUNC-Immuno Plate Maxisorp F96; NalgeNunc International, Rochester, NY, USA) was coated with crude extracts of HDM (10 μ g/ml) or *M. pachydermatis* (4 μ g/ml) diluted with phosphate buffered saline (PBS) and

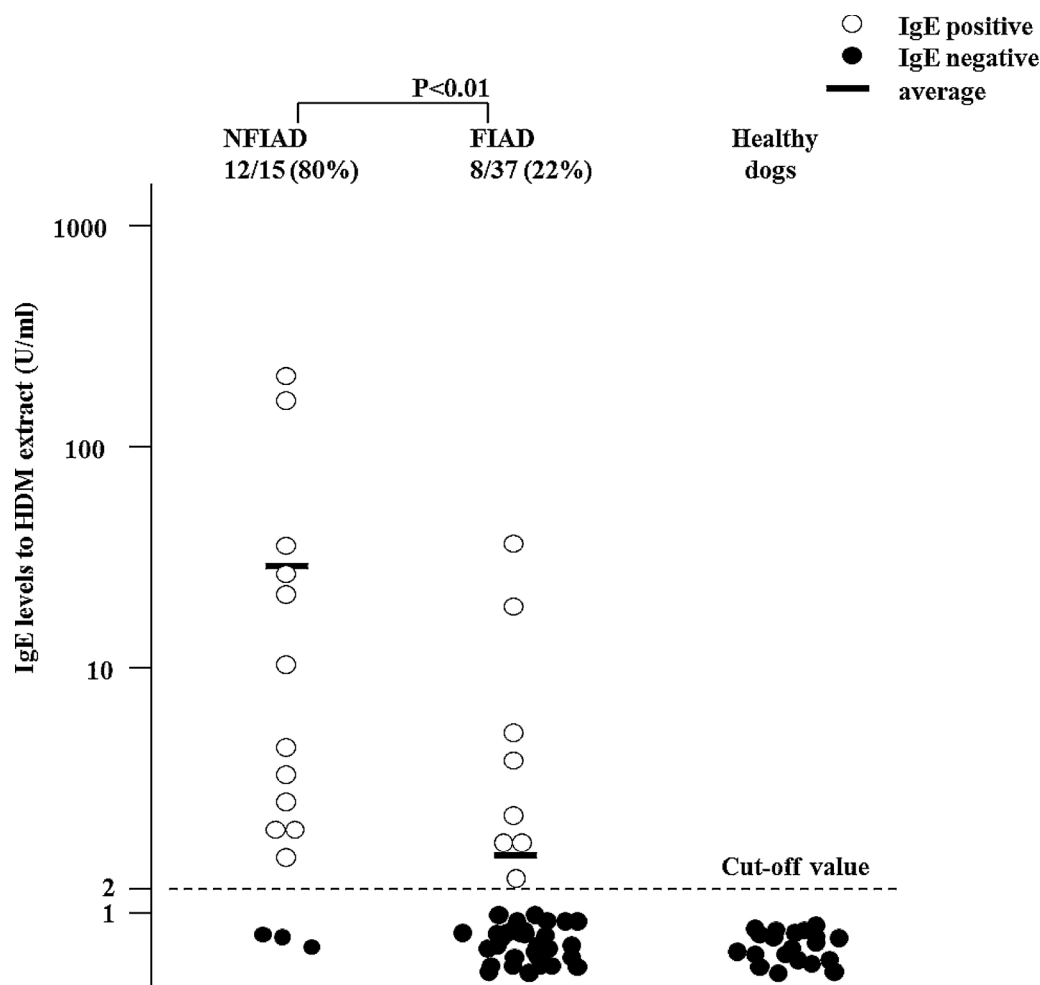


Fig. 2. Specific IgE against HDM extract in NFIAD and FIAD groups and healthy dogs. The cut-off value was the average $\pm$ 3XSD of the IgE level of 20 healthy dogs.

was incubated at 4 °C overnight. After washing, several concentrations of serum diluted with 10% fetal calf serum (FCS) with PBS with Tween 20 (FCS-PBS-T) were added to the wells and the plate was incubated for 3 h at room temperature. The plate was washed, and incubated at 4 °C overnight with a mouse monoclonal anti-dog IgE antibody (0.5 μ g/ml) (DeBoer et al., 1993) diluted with FCS-PBS-T. After washing, biotinylated rat monoclonal anti-mouse IgG₁ (Zymed Laboratories, San Francisco, CA, USA) diluted in FCS-PBS-T was added. The plate was incubated for 1 h at room temperature. After washing, β -D-galactosidase-conjugated streptavidin (Zymed Laboratories) diluted in 1% bovine serum albumin (BSA) in PBS with Tween 20 was added. The plate was incubated for 1 h at room temperature. After washing, 0.1 mM 4-methylumbelliferyl- β -D-galactopyranoside (Sigma-Aldrich, St Louis, MO, USA) diluted in Buffer A (10 mM Na₂HPO₄, 1 mM MgCl₂, 10 mM NaCl, 0.02% NaN₃, and 0.1% BSA) was added to each well. The plate was incubated for 2 h at 37 °C. The enzyme reaction was stopped with 0.1 M glycine-NaOH (pH 10.2), and the fluorescence intensity was read as fluorescence units (FU) on a microplate fluorescence reader (Fluoroskan, Flow Laboratories, McLean, VA, USA). The standard curve was set by positive sera. The cut-off was set as the value above the mean plus 3 standard deviation (SD) from the sera of 20 healthy dogs that were tested concurrently.

2.4. IDST to *M. pachydermatis* and HDM extracts

IDST was performed to subject dogs according to the protocol from Fujimura and Sakaguchi (2005). The *M. pachydermatis* and HDM (*D.*

farinae and *D. pteronyssinus*) allergens were diluted to a concentration of $\times 10$ -500 with the diluent containing 0.9% NaCl and 0.4% phenol. The diluent was served as the negative control whereas a histamine solution (0.0275 mg/ml) served as the positive control. Using a test syringe with a 26-gauge needle, 0.05 mL solutions of the allergens and the controls were injected intradermally into clipped skin on the ventro-lateral thorax. After 15-20 min of the injection, the diameter of wheals was measured.

The results of IDST with those of a fluorometric ELISA in dogs against both antigens were compared by calculating sensitivity, specificity, positive predictive value, negative predictive value and accuracy.

2.5. Detection of allergen-specific IgE by immunoblotting analysis

M. pachydermatis extracts were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were electrotransferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA), as described elsewhere (Kubota et al., 2012). After blocking with PBS containing 5% non-fat dried milk, the membrane was incubated with dog serum (a pool of samples positive for anti- *M. pachydermatis* IgE in ELISA) diluted 1:10 with PBS containing 0.05% Tween-20 (PBS-T) and 5% non-fat dried milk for 1 h. After washing, mouse anti-dog IgE (0.5 μ g/ml) (DeBoer et al., 1993) diluted with PBS-T containing 5% non-fat dried milk was added on the membrane and incubated overnight at 4 °C. After washing, horseradish peroxidase-conjugated goat anti-mouse IgG (0.05 μ g/ml) (Bio-Rad Laboratories) diluted with PBS-T containing 5% non-fat dried milk was

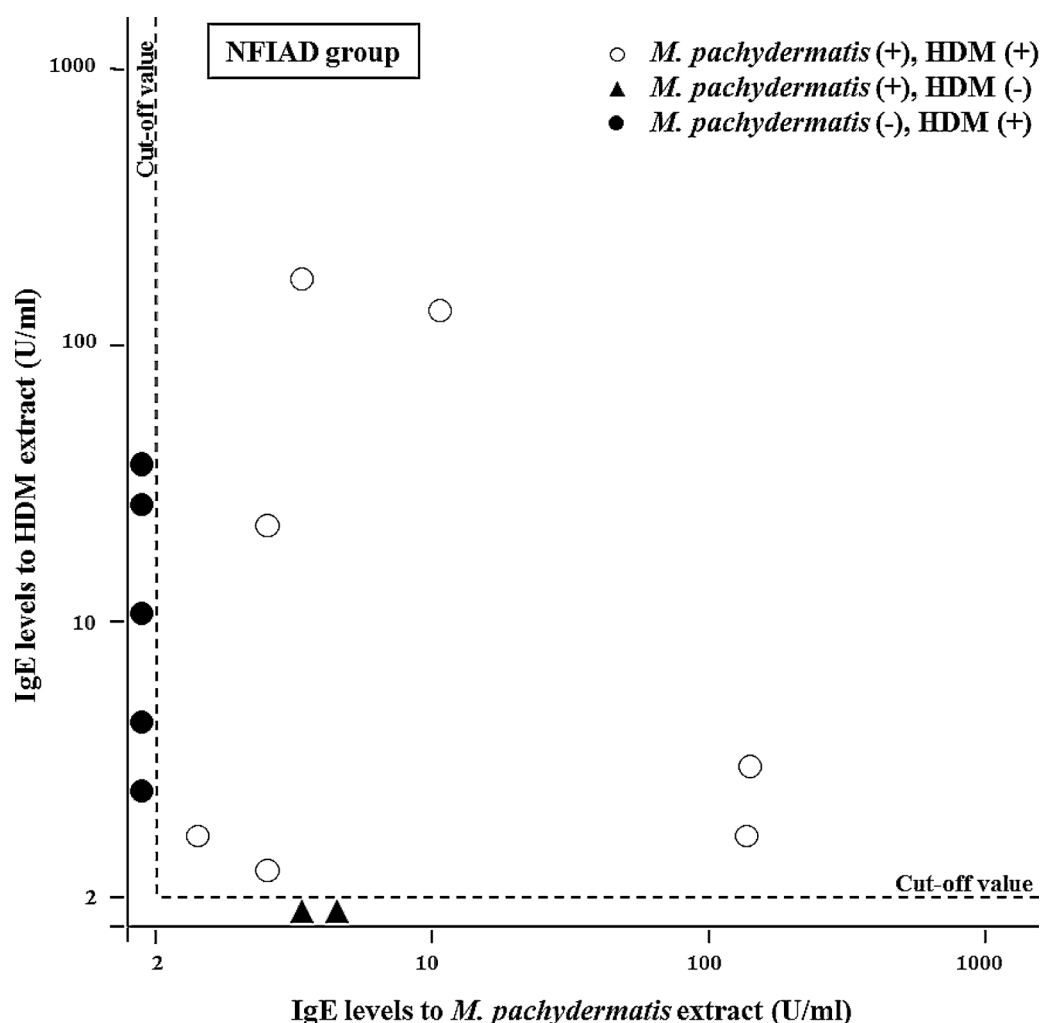


Fig. 3. Comparison between IgE against *M. pachydermatis* and that against HDM extract in NFIAD group.

Open circle indicates dog with specific IgE against *M. pachydermatis* and HDM; Filled triangle indicates dogs with specific IgE against *M. pachydermatis*; Filled circle indicates dogs with specific IgE against HDM.

added on the membrane and incubated for 1 h. After washing, the membrane was incubated with enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ, USA) and was analyzed using ImageQuant LAS 4000 min. (Fujifilm, Tokyo, Japan).

2.6. Statistical analysis

Statistical analyses were performed using Mann-Whitney U-test, Fisher's exact test, and Spearman's rank correlation coefficient. Statistical significance was defined as $P < 0.05$ (Statcel Ver. 2, OMS publishing Inc., Tokyo, Japan).

3. Results

3.1. IgE against *M. pachydermatis* extract in NFIAD and FIAD groups

As shown in Fig. 1, 9 (60%) of the 15 dogs in NFIAD group and 6 (16%) of the 37 dogs in FIAD group had specific IgE against *M. pachydermatis* above the cut-off value. The dogs in NFIAD group showed significantly specific IgE against *M. pachydermatis* than the dogs in FIAD group (Mann-Whitney U-test, $P < 0.01$). Two dogs in NFIAD group showed very high levels of IgE against *M. pachydermatis*.

3.2. IgE against crude HDM extract in NFIAD and FIAD groups in comparison with results for *M. pachydermatis*

As shown in Fig. 2, 12 (80%) of the 15 dogs in NFIAD group and 8 (22%) of the 37 dogs in FIAD group had specific IgE against HDM. The dogs in NFIAD group showed significantly specific IgE against HDM compared with dogs in FIAD group (Mann-Whitney U-test, $P < 0.01$). Moreover, 7 of the 15 dogs in NFIAD group had specific IgE against both *M. pachydermatis* and HDM (Fig. 3), but there was no significant correlation between the levels of specific IgE against the two antigens (Spearman's rank correlation coefficient, $r_s = 0.25$). In contrast, 3 of 37 dogs in FIAD group had specific IgE against both *M. pachydermatis* and HDM (Fig. 4).

3.3. IDST in NFIAD and FIAD groups

All 52 dogs with CAD underwent IDST for crude *M. pachydermatis* and HDM extracts. The positive rates in NFIAD and FIAD groups were 73% and 16%, respectively, for *M. pachydermatis* extract and 80% and 8%, respectively, for HDM extract (Table 1). For both *M. pachydermatis* and HDM extracts, the NFIAD group showed significantly higher positive rate than FIAD group (Mann-Whitney U-test, $p < 0.01$) (Table 1).

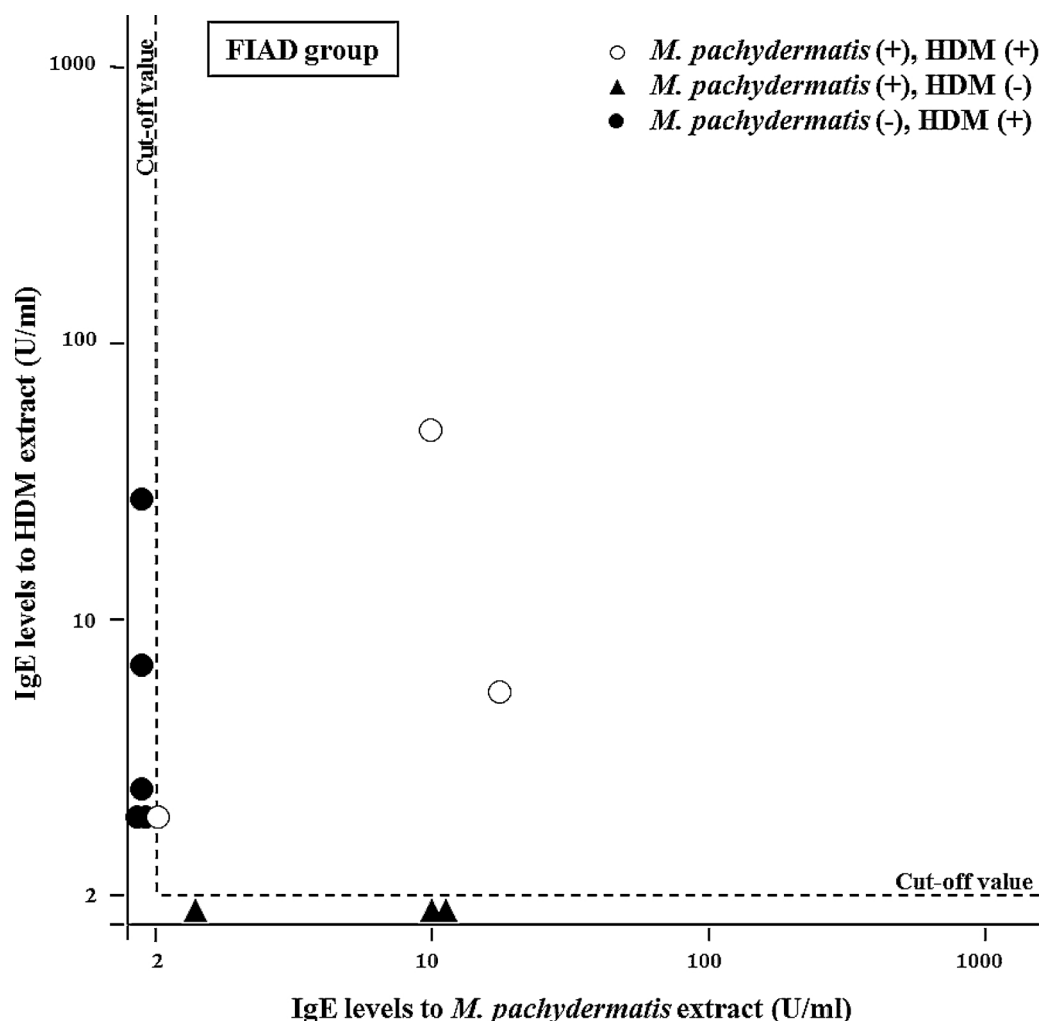


Fig. 4. Comparison between IgE against *M. pachydermatis* and that against HDM extract in FIAD group.

Open circle indicates dog with specific IgE against *M. pachydermatis* and HDM; Filled triangle indicates dogs with specific IgE against *M. pachydermatis*; Filled circle indicates dogs with specific IgE against HDM.

Table 1

The IDST in NFIAD and FIAD groups.

Allergen	NFIAD		FIAD	
	n ^a	% ^b	n	%
<i>M. pachydermatis</i>	11/15	73	6/37	16
HDM	12/15	80	3/37	8

^a Positive dogs/Total number of dogs.

^b Positive rate.

Table 2

Association between results of ELISA and IDST for *M. pachydermatis* extract.

		IDST	
		+	-
ELISA	+	9 ^a	6
	-	8	29

Sensitivity: 53%.

Specificity: 83%.

Positive predictive value: 60%.

Negative predictive value: 78%.

Accuracy: 73% (p < 0.05).

^a Number of dogs.

Table 3

Association between results of ELISA and IDST for HDM extract.

		IDST	
		+	-
ELISA	+	13 ^a	6
	-	2	31

Sensitivity: 87%.

Specificity: 84%.

Positive predictive value: 68%.

Negative predictive value: 94%.

Accuracy: 85% (p < 0.01).

^a Number of dogs.

3.4. Association between results of ELISA and IDST

The results of ELISA and IDST were compared using both NFIAD and FIAD groups. For *M. pachydermatis*, the sensitivity, specificity, positive predictive value, negative predictive value was 53%, 83%, 60% and 78% respectively. Moreover, 38 of the 52 dogs showed the same results and the accuracy was 73% between ELISA and IDST (Fisher's exact test, p < 0.05) (Table 2). For HDM, the sensitivity, specificity, positive predictive value, negative predictive value was 87%, 84%, 68% and 94% respectively. Moreover, 44 of the 52 dogs showed the same results

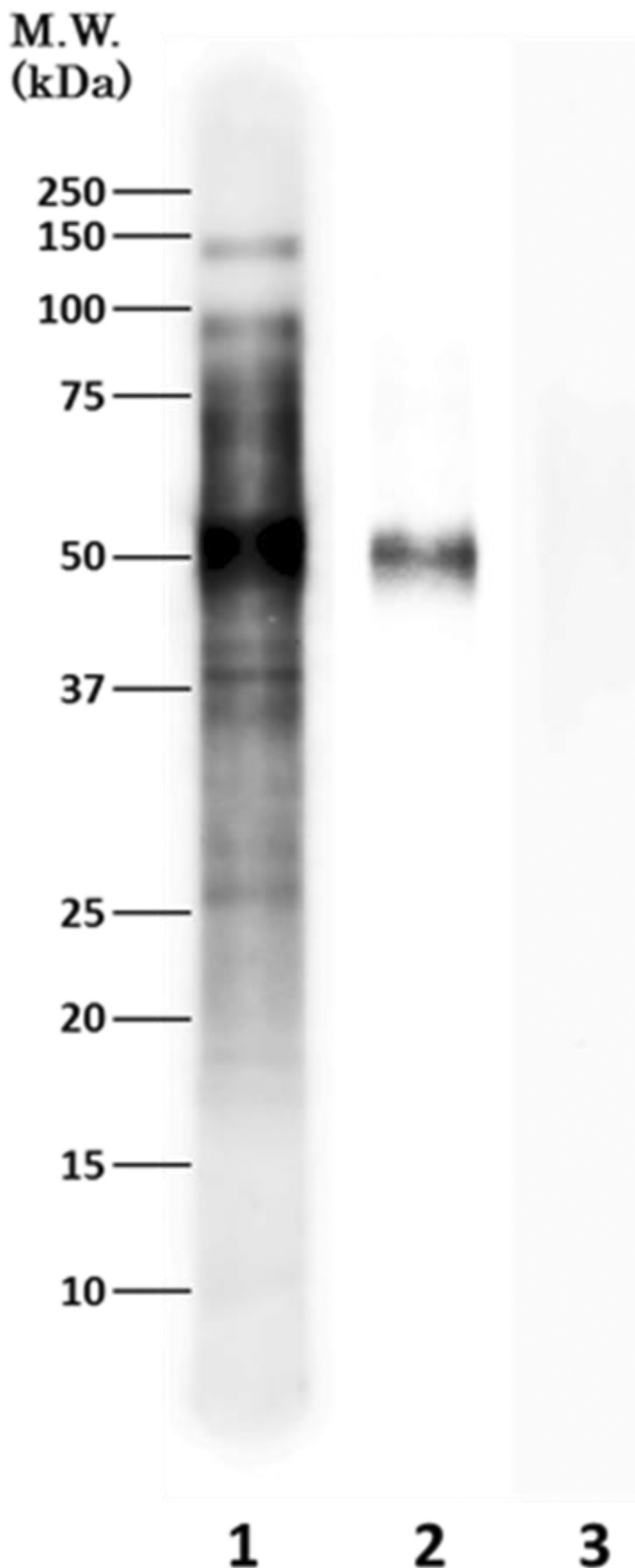


Fig. 5. Immunoblotting analysis of *M. pachydermatis* extract. *M. pachydermatis* extract was electrophoresed by SDS-PAGE and then electrotransferred to PVDF membrane. Lane 1: pooled serum of 4 dogs with anti-*M. pachydermatis* extract IgE, Lane 2: Lowering detection sensitivity of lane 1, Lane 3: pooled serum of 3 dogs with no anti-*M. pachydermatis* extract as negative controls. Precision Plus Protein Standards was used for a molecular weight standard, which is shown on the leftmost column.

and the accuracy was 85% between ELISA and IDST (Fisher's exact test, $p < 0.01$) (Table 3).

3.5. Detection of *M. pachydermatis* by immunoblotting analysis

The results of immunoblotting analysis with pooled sera known to be positive for anti-*M. pachydermatis* IgE on ELISA are shown in Fig. 4. Several positive bands were visible, with the strongest band at approximately 50 kDa molecular weight (Fig. 5).

4. Discussion

The presence of elevated levels of IgE against *Malassezia* in patients with atopic dermatitis was first reported in 1991 (Johansson and Karlström, 1991). *M. sympodialis* and *M. furfur* are commonly found in the upper dermis of healthy humans but their numbers increase in patients with atopic dermatitis (Johansson et al., 2004). Dogs with CAD have higher levels of IgE against *M. pachydermatis* compared with healthy dogs (Nuttall and Halliwell, 2001; Oldenhoff et al., 2014), and dogs with *Malassezia* dermatitis have increased positive reactivity to *M. pachydermatis* extract on IDST compared with dogs without *Malassezia* dermatitis (Farver et al., 2005). To investigate the association between CAD and *Malassezia* in detail, dogs were divided into NFIAD and FIAD groups in the present study. ELISA was used to measure specific IgE against *M. pachydermatis* and IDST was used to examine for the presence of type I hypersensitivity against the antigens *in vivo*. Compared with the FIAD, the dogs with NFIAD were more likely to be positive for *M. pachydermatis*-specific IgE and had higher rates of positive IDST. These results indicated that dogs with NFIAD have more of a tendency to become sensitized to *M. pachydermatis*, even if they have similar clinical signs with the dog with FIAD. This finding highlights the importance of controlling *Malassezia* dermatitis in dogs with NFIAD to reduce the risk of sensitization to *M. pachydermatis*.

The association between CAD and HDM allergens was also demonstrated in the present study. Similar to the findings of *M. pachydermatis*, dogs with NFIAD were significantly more likely to have HDM-specific IgE and positive IDST than the dogs with FIAD. Furthermore, 80% of the dogs with NFIAD had a positive IDST to HDM, of these, 87% also had HDM-specific IgE (Saridomichelakis et al., 1999). In our previous study, on 16 dogs that showed sensitivity to HDM on both IDST and immunodot analysis, 100% and 62.5% had positive IDST to *D. farinae* and *D. pteronyssinus*, respectively (Masuda et al., 1999). The present results for HDM were consistent with the results of these previous studies (Park et al., 2000) has reported 76.8% accuracy between ELISA and IDST for HDM. Similarly, the present study showed a good accuracy between ELISA and IDST of 73% for *M. pachydermatis* and 85% for HDM. Furthermore, sensitivity and specificity for *M. pachydermatis* were 53% and 83% respectively; sensitivity and specificity for HDM were 87% and 84% respectively. Although the sensitivity for *M. pachydermatis* is somewhat low, it is considered to be a method that can be evaluated sufficiently. These results supported the efficiency of the ELISA used in the present study to detect IgE against *M. pachydermatis* and HDM in dogs with CAD.

In a study of humans with atopic eczema, 51 (53%) of 97 patients had *Malassezia*-specific IgE and 45 (46%) of 97 patients showed specific IgE or positive skin prick test to *Malassezia* extract (Casagrande et al., 2006). Patients with food allergy were also investigated in that study but none had *Malassezia*-specific IgE or a positive skin prick test to *Malassezia* extract (Casagrande et al., 2006). Similar to the results of the human study, the results of the present study showed that the dogs with NFIAD were more likely to have *Malassezia*-specific IgE and positive IDST than the dogs with FIAD. Sensitization to *Malassezia* is thought to occur in both humans and dogs with atopic dermatitis. Selective sensitization in atopic patients might be facilitated by the disrupted skin barrier secondary to chronic inflammation (Casagrande et al., 2006). Whole yeast cells and allergenic components of the yeast are likely to

pass through the disrupted skin barrier and come in contact with antigen-presenting cells, such as Langerhans cells in the epidermis (Buentke et al., 2000, 2001). A HDM antigen such as Der p 1 and Der f 1 categorized protease is reported to disrupt the epidermal skin barrier (Oida et al., 2017; Nakamura et al., 2006). A skin pH in atopic dermatitis is in alkaline conditions in both humans and dogs (Cobiella et al., 2019; Panther and Jacob, 2015). An enzymatic potency of these protease antigens in alkaline conditions damages the cutaneous extracellular matrix and therefore weakens the host defense (Oida et al., 2017). The present study showed co-sensitization to *M. pachydermatis*, the skin-associated microbiome in dogs, and HDM especially in NFIAD group, which may be due to epidermal skin barrier defects of HDM antigen. As for additional information that reinforces the co-sensitization recognized in NFIAD group, 8 of 15 animals (53%) showed a positive reaction to multiple antigens in the IDST. Furthermore, selecting the animals in NFIAD group which sensitized to both *M. pachydermatis* and HDM in either ELISA or IDST evaluation, 7 of 10 animals (70%) showed a positive reaction to other antigens in IDST. These co-sensitized antigens were any of the following: glass, weed, pollen, flea, or cotton linter. On the other hand, compared to NFIAD group, FIAD group showed less sensitization to both *M. pachydermatis* and HDM even though clinical signs in FIAD group are comparable to NFIAD group by pruritus and inflammation. We suppose FIAD group was less likely to establish sensitization because the skin barrier function in FIAD group was not affected by HDM antigen as much as NFIAD group.

Immunoblotting analysis of *M. pachydermatis* extract in the sera of dogs with CAD, identified an allergen in the molecular weight domain of 35–150 kDa, with the strongest reaction detected in the vicinity of 50 kDa. The major allergen of *M. pachydermatis* have been reported to have a molecular weight of 45, 52, 56, and 63 kDa (Chen et al., 2002). Among the major allergens reported in the previous study, a molecular weight of 45, 52, and 56 kDa are detected by the antibody in the present study. Therefore, the allergens detected in the present study were likely to be the major allergens.

5. Conclusions

This study demonstrated IgE reactivity to crude *M. pachydermatis* and HDM allergens in dogs with CAD, and the study showed that dogs with NFIAD were more likely to have IgE against both *M. pachydermatis* and HDM and strong reactivity on IDST for *M. pachydermatis* and HDM extract compared with those with FIAD. The results of this study indicated that dogs with NFIAD had an increased risk for sensitization to *M. pachydermatis* compared with dogs with FIAD. Perhaps co-sensitization occurred due to HDM protease antigens, Der f 1 and/or Der p 1, proteolytic activity related epidermal skin barrier defects. Therefore, it is important to control *Malassezia* dermatitis in dogs with NFIAD to minimize the risk of sensitization to *M. pachydermatis*.

Declaration of Competing Interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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