

Identification of major allergens of *Malassezia pachydermatis* in dogs with atopic dermatitis and *Malassezia* overgrowth

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Abstract We have previously shown that both atopic and normal dogs generate an IgG response to antigens of *Malassezia pachydermatis*. The aim of this study was to compare IgE responses to separated proteins of *M. pachydermatis* in 28 atopic dogs with *Malassezia* dermatitis and 22 clinically normal dogs using Western immunoblotting. Six different detection systems were evaluated in order to assess sensitivity and eliminate non-specific binding and cross-reactivity. The protocol yielding the best results utilized a monoclonal mouse antidog IgE, an alkaline phosphatase conjugated goat antimouse IgG which had been passed through a canine IgG column 3 times, a chemiluminescent substrate and a digital imaging system. Proteins of 45, 52, 56 and 63 kDa were recognized by more than 50% of the atopic dog sera and thus represented major allergens. Only a minority of normal dogs showed faint IgE binding to these proteins. The results indicate that the majority of atopic dogs with *Malassezia* dermatitis have a greater IgE response than normal dogs, suggesting an IgE-mediated immune response may be clinically important in the pathogenesis of the disease.

Keywords: atopic dermatitis, dogs, IgE, *Malassezia*

INTRODUCTION

Malassezia pachydermatis, a lipophilic, unipolar-budding yeast is a commensal on canine skin and it can be isolated from the skin and ear canals of healthy dogs.^{1–6} However, a dermatitis associated with elevated cutaneous populations of *M. pachydermatis* that shows a beneficial response to antifungal therapy has been reported in the last two decades,^{7–9} indicating that the organism is an opportunistic pathogen. Several factors have been suggested to cause overgrowth of the organism, such as alterations in skin surface microclimate and defective host immunity.¹⁰ These factors may be associated with various underlying skin diseases including hypersensitivity disorders, bacterial skin infections, keratinization defects and endocrine diseases.^{9–12}

Atopic dermatitis is one of the most common diseases associated with *Malassezia* overgrowth in dogs, and it has been demonstrated that some atopic dogs carry higher numbers of *M. pachydermatis* on their non-lesional and lesional skin than normal dogs.^{3,13,14} Immediate hypersensitivity responses to intradermal injections of *M. pachydermatis* extracts have been demonstrated in atopic dogs with *Malassezia* overgrowth,¹⁵ suggesting that allergens from the organism may be involved in the pathogenesis of atopic dermatitis.

Previous reports have also shown that atopic dogs have elevated levels of *Malassezia*-specific IgG and IgE¹⁶ in their serum.

In human patients with atopic dermatitis, *Malassezia furfur* is known to be allergenic, and a number of IgE-binding components in the range of 9–100 kDa have been documented.^{17–20} Nine allergens of *Malassezia furfur* (Mal f 1–Mal f 9) have now been sequenced and expressed as recombinant proteins,^{21–25} with molecular weights of 37, 21, 20, 35, 18.2, 17.2, 16.2, 19.2 and 14 kDa, respectively.

We have recently shown that the majority of atopic dogs with *Malassezia* dermatitis have a greater IgG response to the yeast than normal dogs, and that an antigen of 25 kDa appears to be clinically important.²⁶ The aim of this study was to further characterize the humoral immune response to *M. pachydermatis* by comparing IgE responses to separated antigens of the yeast in atopic dogs with *Malassezia* dermatitis and normal dogs.

MATERIALS AND METHODS

Serum samples

Serum samples were collected from 28 atopic dogs with *Malassezia* dermatitis presented to the dermatology clinic at the Royal (Dick) School of Veterinary Studies in Edinburgh. Sera of clinically normal dogs were collected from 22 dogs presented for euthanasia at a rescue centre.

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The diagnosis of atopic dermatitis was based on a combination of consistent history and clinical signs, exclusion of other causes of pruritic skin disease and the presence of at least one positive intradermal skin test reaction. Coat brushings, skin scrapings and trial therapy were used to rule out ectoparasites. A 6-week, home-cooked diet trial was conducted to rule out adverse food reactions. No anti-inflammatory medication was given for at least 3 weeks prior to examination. The intradermal skin tests were performed with 57 allergens using 0.05 mL of each allergen extract on the lateral flank after the dogs were sedated with 0.15 mg kg⁻¹ xylazine intramuscularly and clipped. Histamine (1/100 000 w/v) and diluent consisting of buffered saline with 0.4% phenol as preservative were also injected as positive and negative controls, respectively. Test sites were assessed after 20 min and scored from 0 to +4 by comparison with the controls. Reactions ≥ 2 were considered positive.²⁷

Malassezia overgrowth was diagnosed by microscopic observation of Diff-Quik® (Dade AG, Switzerland) stained tape strips.^{10,13,28} Samples were obtained from the groin, axilla and interdigital web and *Malassezia* overgrowth was characterized as an average of 5 or more *Malassezia* organisms per 400 $\times$ field.^{29,30} Involvement of *Malassezia* in cases of otitis externa was assessed by sampling the external ear canal with a cotton swab and transferring the material to a glass slide prior to heat fixing and staining. The criteria for demonstrating overgrowth were as described for the tape strips.

Normal dogs had no history or clinical signs of skin disease and had no lesions on dermatological examination.

Culture of *M. pachydermatis*

An isolate of *M. pachydermatis* was obtained from the ear canal of a dog with *Malassezia* otitis. The use of a single isolate was deemed appropriate because previous studies have shown that *M. pachydermatis* was the only *Malassezia* species isolated from the skin and external ear canal of dogs with either otitis externa or skin infections, and all isolates of *M. pachydermatis* had similar electrophoretic karyotypes.³¹ The sample was cultured on Sabouraud Dextrose agar (Oxoid, UK) containing 20 mg mL⁻¹ chloramphenicol (Intramy-cetin®, Parke-Davis Veterinary, UK) for 48 h at 37 °C, and the colonies were identified as *M. pachydermatis* by microscopic examination. The colonies were then subcultured onto large numbers 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 NH₄HCO₃ (Sigma,

UK) and protease inhibitors (20 mM ϵ -aminocaproic acid, Sigma, UK; 5 mM ethylenediaminetetra-acetic acid, Sigma, UK; and 1 mM phenylmethylsulphonyl fluoride, BDH, UK).^{15,18} The *Malassezia* colonies in the extraction buffer were then mixed vigorously with an equal volume of glass beads (0.4 mm, 40 mesh, BDH, UK) 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 amount of protein obtained was measured with BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL, USA).

SDS-PAGE and electrophoretic transfer

Gel electrophoresis was performed according to the method of Laemmli³² using 10% separating polyacrylamide gel and 4% stacking gel in a discontinuous buffer system containing 0.025 M Tris (Sigma, UK), 0.2 M Glycine (Fisher Scientific, UK) and 0.1% sodium dodecyl sulphate (Fisher Scientific, UK), pH 8.3. The extract (60 μ g) and molecular weight standards were diluted 1:1 with reducing sample buffer (containing 5% β -Mercaptoethanol, Sigma, UK) and heated at 95 °C for 5 min. The extract was then added to one broad well across the top of the gel and the electrophoresis was run at 200 V for 40 min. Fig. 1 shows the protein profile of *M. pachydermatis* extracts on a 10% separating polyacrylamide gel.

The separated proteins and molecular weight standards were transferred from the gel to a polyvinylidene difluoride microporous membrane (Millipore Immobilon™-P Transfer Membrane, Millipore Corporation, Bedford, MA, USA) in a Bio-Rad Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell according to the manufacturer's instructions. The transfer buffer contained 25 mM Tris (Sigma, UK), 192 mM Glycine (Fisher Scientific, UK) and 20% methanol (Fisher Scientific, UK), pH 8.3. The transfer was run at 80 mA per minigel for 1 h. The quality of transfer was checked by staining gels and molecular weight standards blotted onto the membrane with Coomassie Brilliant Blue R-250 (BDH, UK).

Optimization of methodology to detect IgE binding proteins on the *Malassezia pachydermatis* immunoblots

Various methodologies and reagents were evaluated to detect IgE binding proteins in *M. pachydermatis* (summarized in Table 1). Two methods were investigated to allow the immunoblot membrane to be probed with individual dog sera: cutting the membrane into narrow strips or placing the whole membrane into a multichannel miniblitter (Immunetics Inc., Cambridge, MA, USA). The blocking reagents that were evaluated included 5% skimmed milk, casein solution (Vector Laboratories Inc., Burlingame, CA, USA), Tween 20 (Fisher Scientific, UK), Tween 80 (Sigma, UK), and an avidin/biotin blocking kit (Vector Laboratories Inc., Burlingame, CA, USA). The following IgE-specific reagents were evaluated: a polyclonal epsilon chain

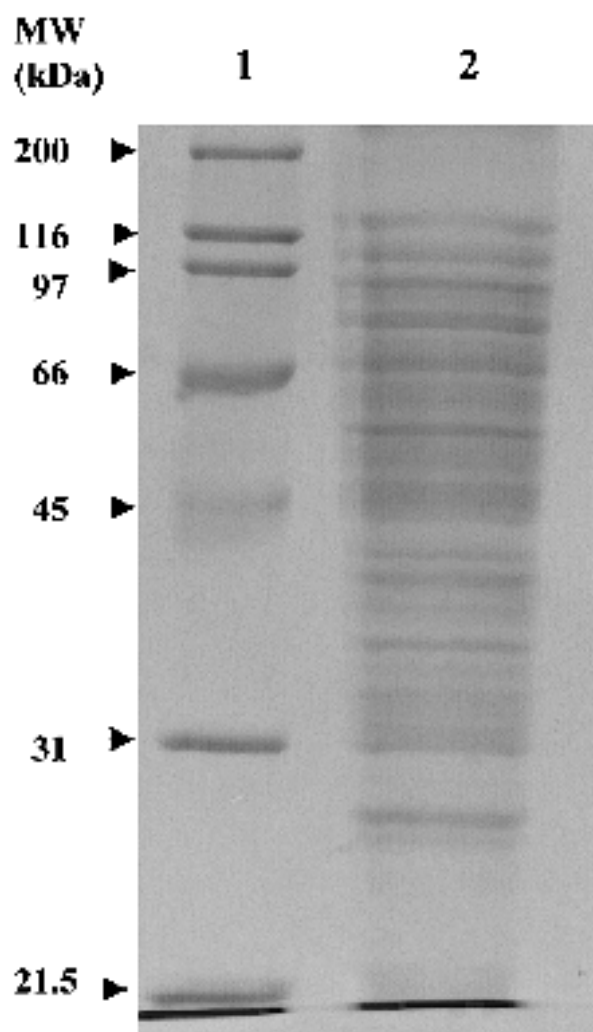


Figure 1. Coomassie Brilliant Blue stained *M. pachydermatis* extracts on a 10% separating polyacrylamide gel. Lane 1: molecular weight markers; lane 2: *Malassezia* extract.

specific goat antidog IgE conjugated with horseradish peroxidase (HRP) (Bethyl Laboratories, Montgomery TX, USA); two monoclonal mouse antidog IgE antibodies (D9#3116, kindly donated by Dr D. J. Deboer, University of Wisconsin, USA and E6-71A1, Custom Monoclonals International, W. Sacramento, CA, USA); and a biotinylated recombinant human IgE receptor alpha chain (kindly donated by Dr C. McCall, Heska Corporation, Fort Collins, USA). For visualization of binding, the following conjugates labelled with either alkaline phosphatase (AP) or HRP were used: a monoclonal mouse antigoat IgG-AP (Sigma, UK); a monoclonal bovine antimouse IgG1-HRP (Serotec, UK); two polyclonal goat antimouse IgG antibodies labelled either with HRP (Sigma, UK) or AP (Southern Biotechnology Associates Inc., USA); and two avidin reagents (Vectastain® Elite ABC kit, Vector Laboratories Inc., Burlingame, CA, USA and Extravidin®-peroxidase, Sigma, UK). Two different detection systems were evaluated: substrate colour change and chemiluminescence. For standard blots, DAB (3,3'-diaminobenzidine, Vector Laboratories Inc., Burlingame,

CA, USA) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma, UK) were used to detect HRP- and AP-labelled reagents, respectively. For chemiluminescent blots, ECL™ (Amersham Pharmacia Biotech, UK) was used for HRP-labelled reagents and CDP-Star™ (Tropix Inc., Bedford, USA) for AP-labelled reagents (Table 1).

Assessment of reagent specificity

Purified dog IgG (Sigma, UK), dog IgE (Bethyl Laboratories, Montgomery TX, USA) and mouse IgG1κ (Sigma, UK) were used to test the specificity of reagents. The purified antibodies were diluted 1:1 with nonreducing sample buffer (in view of the sensitivity of dog IgE to 2-mercaptoethanol³³), and the gel electrophoresis performed as described above. They were then transferred from the gel to a membrane, blocked with 5% skimmed milk in Tris-buffered saline containing 0.02 M Tris, 0.5 M NaCl and 0.1% Tween 20 (TTBS), pH 7.5 for 1 h and sequentially incubated with the various combinations of IgE-specific reagents and secondary antibody conjugates summarized in Table 1.

The antibody conjugate used in protocol 6 had previously been shown to exhibit cross-reactivity between mouse IgG1 and canine IgG (data not shown). To eliminate this cross-reactivity, the conjugate had been further purified using affinity chromatography. Briefly, purified dog IgG (10 mg) dissolved in 0.15 M NaCl (Sigma, UK) was exchanged with sodium hydrogen carbonate buffer (0.2 M NaHCO₃ and 0.5 M NaCl, pH 8.5) using HiTrap Desalting (Amersham Pharmacia Biotech, UK) and coupled to cyanogen bromide (CNBr)-activated Sepharose™ 4B (Amersham Pharmacia Biotech, UK). The remaining active sites in the Sepharose were blocked with 1 M ethanolamine (Sigma, UK), and the gel was then packed into a column. The AP-labelled goat antimouse IgG (Southern Biotechnology Associates Inc., USA) was then passed through the canine IgG column on three occasions using an FPLC® system (Amersham Pharmacia Biotech, UK). Antibodies that did not bind to canine IgG were collected. The specificity of solution passed through the column was checked after each run using the method described above. After three passages through the column, the AP-labelled goat antimouse IgG did not bind to either dog IgE without the presence of monoclonal mouse antidog IgE or to dog IgG with or without the presence of antidog IgE antibody (Fig. 2). For long-term storage, the final solution collected was concentrated using Centrplus™ concentrators (Amicon Inc., Beverly, USA) and the antibody concentration was measured with a spectrophotometer. The concentrated solution was diluted 1:1 in glycerol (BDH, UK) to a final concentration of 0.29 mg mL⁻¹.

Immunoblotting

Evaluation of the various reagents and protocols revealed that an immunoblot membrane cut into strips and protocol 6 (Table 1) yielded the best result. The miniblitter did not produce repeatable results, and

Table 1. Methodologies and reagents evaluated in this study to detect IgE binding proteins in *M. pachydermatis*

Protocol	Reagents				
	IgE-specific Reagent	Secondary developing reagent	Substrate	Detection	Comments
1	Goat antidog IgE-epsilon chain specific, HRP (Bethyl Laboratories)		DAB	Colour change on blots	Abandoned due to lack of sensitivity
2	Goat antidog IgE-epsilon chain specific, HRP (Bethyl Laboratories)	Monoclonal antigoat IgG, AP (Sigma)	BCIP/NBT	Colour change on blots	Abandoned due to lack of sensitivity
3	Monoclonal mouse antidog IgE (Dr D.J. Deboer)	Goat antimouse IgG, HRP (Sigma)	ECL TM	Chemiluminescence detected on X-ray films (Hyperfilm TM ECL TM , Amersham Life Science)	Abandoned due to cross-reactivity of the secondary antibody with canine IgG
4	Monoclonal mouse antidog IgE (Dr D.J. Deboer)	Bovine antimouse IgG1, HRP (Serotec)	ECL TM	Chemiluminescence detected on X-ray films	Abandoned due to cross-reactivity of the secondary antibody with canine IgG
5	Biotinylated recombinant human IgE receptor alpha chain (Heska)	Vectastain® Elite ABC kit (Vector Laboratories) Extravidin®-peroxidase (Sigma)	ECL TM	Chemiluminescence detected on X-ray films	Abandoned due to cross-reactivity of the secondary-developing reagents with <i>Malassezia</i> extracts
6	Monoclonal mouse antidog IgE E6–71A1 (Custom Monoclonals International)	Goat antimouse IgG1, AP (Southern Biotechnology Associates) before and after passing through a canine IgG column three times	CDP-Star TM	Chemiluminescence detected using Kodak Digital Science TM Image Station 440CF	Successful

HRP: horseradish peroxidase; AP: alkaline phosphatase; DAB: 3,3'-diaminobenzidine; BCIP/NBT: 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

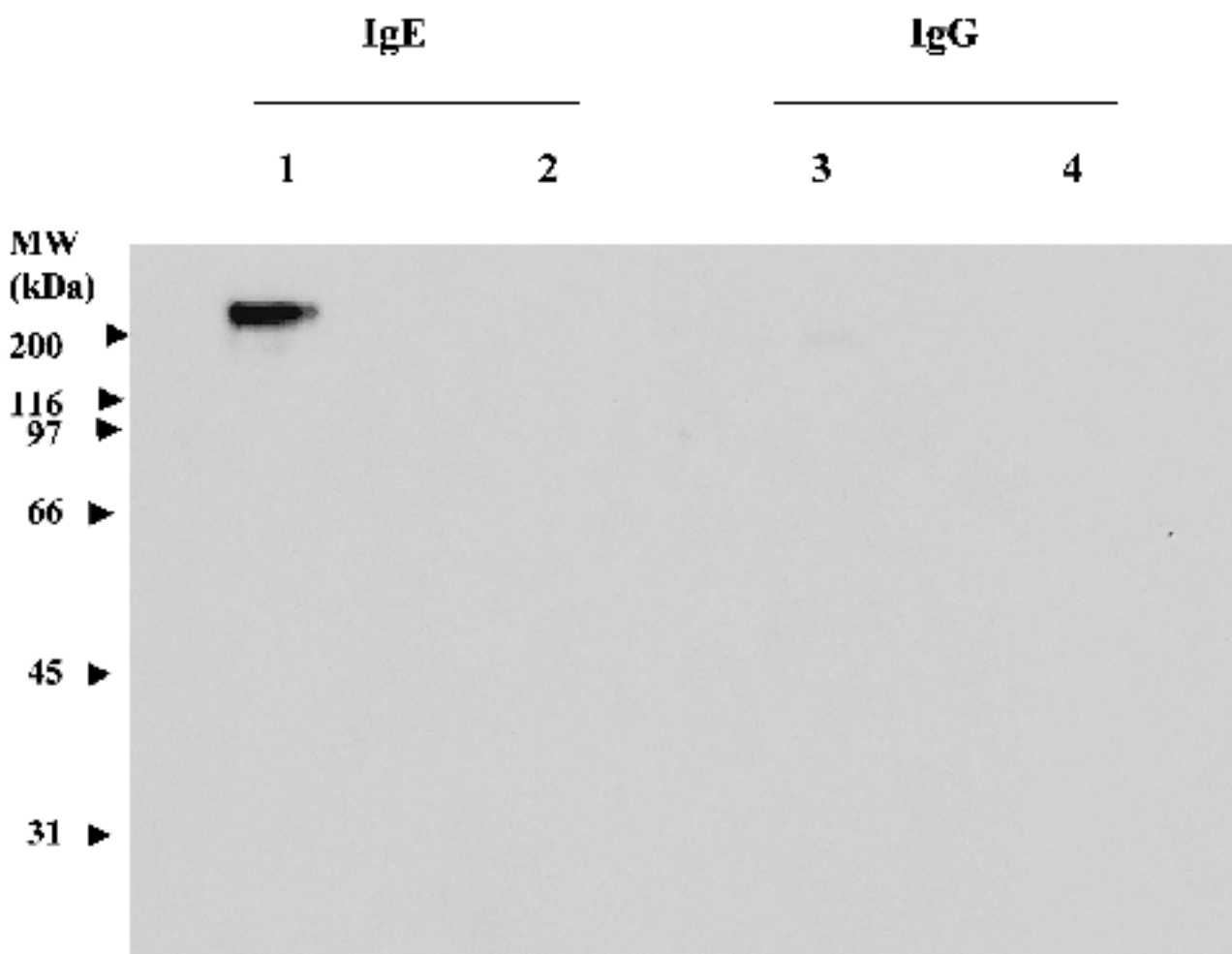


Figure 2. Examination of reagent specificity in protocol 6 to detect IgE-binding proteins in *Malassezia pachydermatis*. Purified dog IgE (lanes 1 and 2) and IgG (lanes 3 and 4) were diluted with nonreducing sample buffer, separated in 10% SDS-PAGE (0.2 µg per lane), and transferred to a membrane. The membranes were then sequentially incubated with monoclonal mouse antidog IgE (E6-71A1) diluted 1:500 in dilution buffer (1% skimmed milk in TTBS), the secondary developing reagent which had been passed through a canine IgG column three times (1:2000 dilution) and the substrate (lanes 1 and 3). For the control groups (lanes 2 and 4), the antidog IgE antibody was replaced with the dilution buffer.

protocols 1–5 were rejected due to lack of sensitivity or specificity. The individual serum samples were therefore analysed using the following conditions. The strips were blocked for 1 h with 5% skimmed milk in TTBS. After washing with TTBS for 15 min (3×5 minute rinses), each strip was incubated with individual dog serum diluted 1:5 in dilution buffer (1% skimmed milk in TTBS) for 1 h. The strips were then washed again in TTBS for a further 15 min. Bound IgE was detected by incubating the strips for 1 h with monoclonal mouse antidog IgE E6-71A1 (diluted 1:500). After further washing with TTBS for 15 min, the strips were incubated with AP-labelled goat antimouse IgG1 (diluted 1:2000) for 1 h. The strips were washed again with TTBS for 15 min, and then rinsed with detection buffer containing 0.1 M Tris-HCl, 0.1 M NaCl and 5 mM $MgCl_2$ (BDH, UK), pH 9.5. The strips were developed by incubating them with a chemiluminescent substrate, CDP-StarTM, for 5 min. After draining off excess liquid, the strips were wrapped in cling film and placed on a Kodak Digital ScienceTM Image Station 440CF along with Coomassie blue stained molecular weight

markers. The membrane was exposed for 15 min, and blots were visualized on the computer screen. Kodak Digital ScienceTM 1D Image Analysis Software was used to detect the bands on the blots and to determine the molecular weight of bands by analysing their positions relative to molecular weight markers.

To confirm that the major bands on the blots represented IgE-binding proteins, a pooled serum sample was generated from all the atopic dogs and from all the normal dogs. The pooled samples were analysed by SDS-PAGE and immunoblotting as described above both before and after heating to 56 °C for 2, 4 and 8 h.

RESULTS

Immunoblotting with individual dog sera

The IgE immunoblots of individual dog sera from the two groups are shown in Fig. 3(a and b). The intensity of binding seen in normal dogs (Fig. 3a) was much weaker than that seen in the atopic dogs with *Malassezia* dermatitis (Fig. 3b).

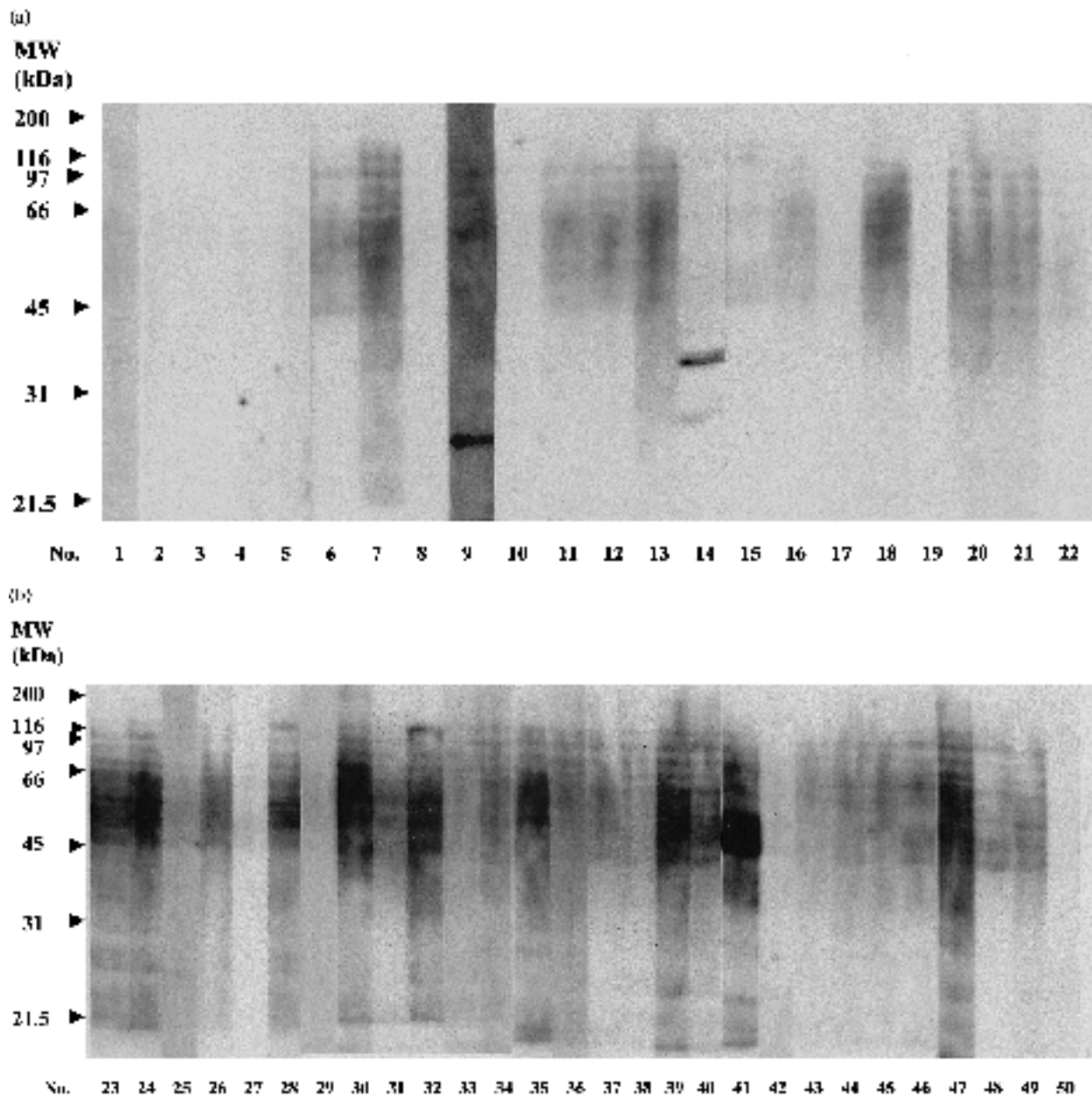


Figure 3. IgE-binding proteins in extracts of *M. pachydermatis* detected by immunoblotting with dog sera from 2 groups: (a) normal dogs; and (b) atopic dogs with *Malassezia* dermatitis. The images of blots were captured by a digital imaging station and printed out directly from the computer. The numbers along the bottom signify the strips probed with individual dog sera. The molecular weight markers on the left are 200, 116, 97, 66, 45, 31 and 21.5 kDa.

The intensity of binding in atopic dogs with *Malassezia* dermatitis was strongest between 45 and 63 kDa. IgE binding proteins with molecular weights of 45 (61% of dogs), 52 (50%), 56 (61%) and 63 (68%) kDa were recognized in more than 50% of the dogs (Fig. 4). Strong bands were also detected at 26 (29%), 48 (39%) and 60 (18%) kDa.

Approximately one-third of the normal dogs showed faint binding to proteins between 45 and 63 kDa, but clearer bands at 45 (in 18% of dogs), 52 (36%), 56 (32%) and 63 kDa (36%) were detected by the image analysis software (Fig. 4). Furthermore, one dog had a prominent band at 26 kDa (No. 9) (Fig. 3a).

In order to confirm that the bands detected on the blots were IgE-binding proteins, a pooled serum sample was prepared from the atopic dogs with

Malassezia dermatitis and from the normal dogs. The pooled serum samples were heat treated at 56 °C for 2, 4 and 8 h to detect the heat lability of binding reactions (Fig. 5). The binding patterns seen in the two serum pools were very similar to those seen in the corresponding individual serum samples. As with the individual blots, the binding intensity seen in the atopic dog serum pool was much stronger than that in the normal dogs. Between 45 and 63 kDa, the binding intensity declined dramatically over the period when serum pools were heated. Gradually decreasing binding activity was also seen on one single band at 26 kDa. Accordingly, bands of 26 kDa and any binding detected between 45 and 63 kDa on the strips from the individual dogs were regarded as IgE specific.

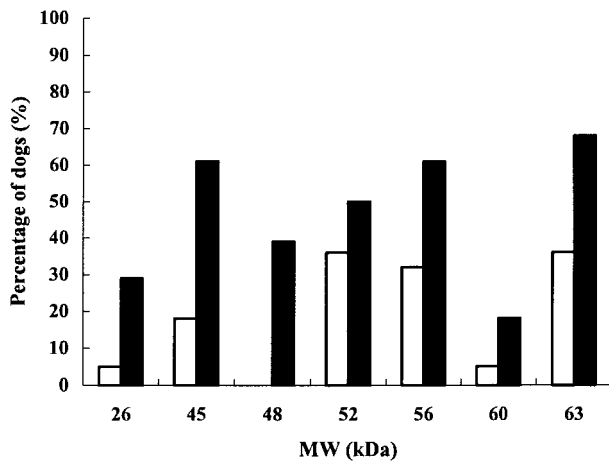


Figure 4. The percentage of dogs from the two groups showing IgE binding to individual proteins in extracts of *M. pachydermatis*. The bands on the blots shown in Fig. 3 were detected using Kodak Digital Science™ 1D Image Analysis Software and the molecular weight of bands generated by the software was used to calculate the percentage of dogs reacting to individual bands. Filled columns: atopic dogs with *Malassezia* dermatitis; open columns: normal dogs. x-axis: molecular weights of the bands detected on the strips; y-axis: percentage of dogs reacting to those bands.

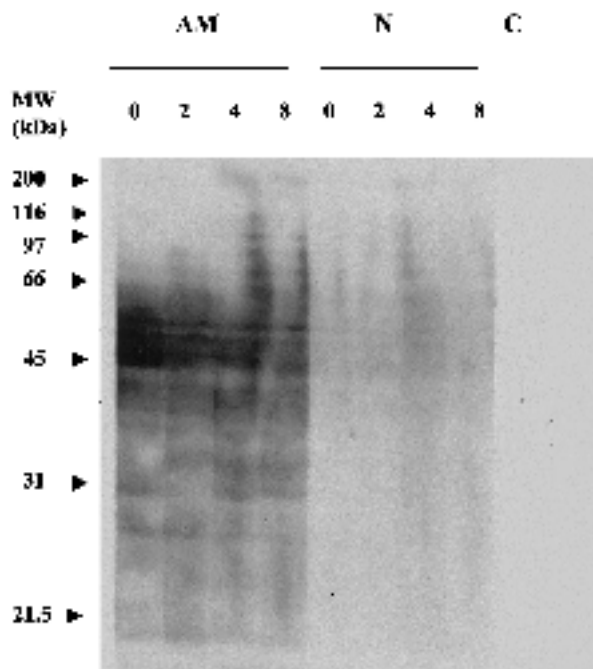


Figure 5. Heat-lability of binding detected in two serum pools: one from atopic dogs with *Malassezia* dermatitis and one from normal dogs. AM: atopic dogs with *Malassezia* dermatitis; N: normal dogs; C: control (dilution buffer: 1% skimmed milk in TTBS). The numbers represent the length of heat treatment. 0: nonheated serum pool; 2, 4, and 8: after heating for 2, 4 and 8 h, respectively. Note the decrease in staining intensity seen in bands at 26 and 45–63 kDa after heating.

DISCUSSION

In this study, we have shown that *Malassezia* dermatitis in atopic dogs is associated with an IgE response to the organism (compare Fig. 3a and b). This result con-

firms the findings of previous studies that have demonstrated elevated concentrations of *Malassezia*-specific IgE in atopic dogs with *Malassezia* overgrowth by ELISA¹⁶ and positive responses following intradermal injection of *Malassezia* extracts.¹⁵ Furthermore, we have demonstrated that the IgE response is predominantly targeted against proteins with molecular weights of 45, 52, 56 and 63 kDa. As these proteins were recognized in over 50% of the atopic dogs with *Malassezia* dermatitis, but only recognized by a minority of normal dog sera, they can be classified as major allergens. Strong bands were also detected with molecular weights of 26, 48 and 60 kDa, but as these were recognized in less than half of the atopic dogs they can be regarded as minor allergens. Weak IgE binding was also seen in some normal dogs between 45 and 63 kDa. The cause of this weak IgE binding in normal dogs is unknown. It has been shown that extracts of *M. pachydermatis* at high concentrations are capable of inducing wheal and flare responses in normal dogs.¹⁵ This could be an irritant reaction or an IgE response present to commensal populations of the yeast. The latter is certainly the case with IgG responses as shown in previous studies.^{16,26}

Of all the allergens detected, those with molecular weights of 45 and 48 kDa seemed to be the most relevant because they were recognized by most atopic dogs with *Malassezia* overgrowth but only a few normal dogs (Fig. 4). Further studies are required to determine the identity of these proteins using protein purification and N-terminal sequencing. They may represent two different proteins or a single protein with variable glycosylation.

In contrast to the low molecular weight allergens of *Malassezia furfur* (Mal f 1–Mal f 9) recognized by humans, dogs recognized higher molecular weight allergens in this study. Similar findings have been documented with *Dermatophagoides* allergens. Most human atopic sera have high IgE titres to group 1 and group 2 allergens of *Dermatophagoides*^{34,35} which are low molecular weight proteins, whereas in dogs the most important *Dermatophagoides* allergens are high molecular weight proteins greater than 90 kDa.^{36–38}

Whilst optimizing the methodology to detect IgE binding proteins in *M. pachydermatis*, we had to overcome problems of lack of sensitivity and specificity (summarized in Table 1). Protocols 1 and 2 were rejected because there were no visible bands, even with the amplification step introduced in protocol 2. To increase the sensitivity, various chemiluminescent protocols were investigated (protocols 3–6). Luminescent detection methods are believed to provide several advantages over traditional chromogenic procedures, such as generally increasing the sensitivity of both HRP and AP systems, rapid detection and long-lasting signals permitting multiple exposures.³⁹ To increase the specificity of the chemiluminescent methods, monoclonal antibodies and an IgE receptor reagent were used. In protocols 3 and 4, the secondary antibodies cross-reacted with canine IgG and they were

abandoned due to lack of specificity. This problem is confounded by the fact that levels of allergen-reactive IgG may be several thousandfold higher than levels of IgE in normal and atopic animals.⁴⁰ The results of our previous study obtained using a less sensitive substrate (DAB) to detect *Malassezia*-specific IgG verified that the levels of antigen-specific IgG were much higher than levels of IgE. The high levels of *Malassezia*-specific IgG in dog sera could enhance the IgG signals and eclipse the IgE reaction.

The high-affinity receptor for IgE, FcεRI, is a requisite for the IgE-mediated immediate hypersensitivity reaction and the interaction between IgE and FcεRI is highly specific.⁴¹ In spite of offering absolute specificity for IgE, the FcεRI receptor reagent used in protocol 5 was not successful because the avidin reagents bound to *Malassezia* proteins. This could stem from endogenous protein-bound biotin or lectins in the extracts, as these contained a crude mixture of both cytoplasmic and cell wall components.

In protocol 6, a combination of reagents that had previously been used successfully to detect IgE binding in ELISA assays (unpublished observations) was assessed. In this protocol, the cross-reactivity of the secondary antibody was removed by immunoabsorption through a canine IgG column. Although protocol 6 proved to be successful, it is possible that the use of the cross-absorbed secondary reagent with other primary antibodies could also be suitable. With this protocol, we also used a digital imager to resolve the bands, rather than assessment of X-ray films. We found that X-ray films produced previously reported problems such as weak bands being overshadowed by adjacent strong signals, broad diffuse images resulting from overexposure and film saturation. We found that the image station and image analysis software provided better resolution of the bands detected and enabled more accurate determination of their molecular weights than traditional chemiluminescent methods. It is important to note that chemiluminescent blots are not visible to the naked eye and can only be visualized on X-ray film or on a computer screen (when using digital imaging). Furthermore, the bands on chemiluminescent blots are not as clear and distinct as those seen on normal chromogenic blots. However, the use of imaging software allows the bands to be resolved without investigator bias.

Despite the various problems associated with lack of specificity, we believe that there are three lines of evidence that confirm that we have detected *Malassezia*-specific IgE. First, using protocol 6 we demonstrated that the reagents did not cross-react with canine IgG (see Fig. 2). Secondly, the IgE binding to the major allergens was shown to be heat labile (Fig. 5). Thirdly, the antigens detected in this study were different from those detected in our previous study using reagents to detect IgG.

In summary, we have demonstrated that *M. pachydermatis* allergens of 45, 52, 56 and 63 kDa appear to be clinically relevant in atopic dogs. However, further

characterization of these allergens is needed before they could potentially be used in intradermal testing or allergen immunotherapy in affected dogs.

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Résumé Nous avons démontré précédemment que les chiens atopiques comme les chiens normaux synthétisaient des IgG en réponse à un stimulus par des antigènes de *Malassezia pachydermatis*. Le but de cette étude était de comparer la synthèse d'IgE vis à vis de différentes protéines de *M. pachydermatis* chez 28 chiens atopiques présentant une dermatite à *Malassezia* et chez 22 chiens cliniquement sains. Six systèmes différents de détection ont été évalués afin d'améliorer la sensibilité et d'éliminer les réactions non spécifiques ou les réactivités croisées. Le protocole donnant les meilleurs résultats était celui avec un antiIgE monoclonal de souris, un système de phosphatases alcalines conjuguées avec des IgG antisouris de chèvre, qui ont été passées trois fois sur une colonne d'IgG canines, un substrat de chimioluminescence et un système d'imagerie digitale. Des protéines de 45, 52, 56 et 63 kDa ont été reconnues par plus de 50% des serums de chiens atopiques, et doivent donc considérées comme des allergènes majeurs. Seule une minorité des chiens normaux ont présenté une réactivité IgE faible à ces protéines. Les résultats de cette étude indiquent que la majorité des chiens souffrant de dermatite à *Malassezia* ont une réponse IgE plus élevée que les chiens normaux, suggérant que la réponse immunitaire médiée par les IgE soit importante cliniquement dans la pathogénie de la maladie.

Resumen En estudios anteriores hemos demostrado que tanto perros atópicos como normales generan una respuesta de IgG a antígenos de *Malassezia pachydermatis*. El objetivo de este estudio fue comparar las respuestas de IgE a proteínas separadas de *M. pachydermatis* en 28 perros atópicos con dermatitis por *Malassezia* y 22 perros clínicamente normales, utilizando Western immunoblotting. Se evaluaron seis sistemas diferentes de detección con el fin de evaluar la sensibilidad y eliminar las uniones inespecíficas y la reactividad cruzada. El protocolo con los mejores resultados utilizaba una IgE monoclonal de ratón anti-canina, una IgG caprina anti-murina conjugada con fosfatasa alcalina pasada por una columna de IgG canina 3 veces, un sustrato quimioluminescente y un sistema de imagen digital. Las proteínas de 45, 52, 56 y 63 kDa fueron reconocidas por más del 50% de sueros de perros atópicos, representando por tanto los principales alérgenos. Sólo una minoría de perros normales mostraban una unión débil de IgE a estas proteínas. Los resultados indican que la mayoría de los perros atópicos con dermatitis por *Malassezia* tienen una respuesta de IgE mayor que los perros normales, sugiriendo que una respuesta inmune mediada por IgE puede ser clínicamente importante en la patogénesis de esta enfermedad.

Zusammenfassung Wir haben in der Vergangenheit nachgewiesen, dass normale Hunde und Hunde mit atopischer Dermatitis gegen *Malassezia pachydermatis*-Antigene eine IgG Antwort entwickeln. Das Ziel dieser Studie war, die mittels Western Blot gemessene IgE Antwort gegen Proteine von *M. pachydermatis* bei 28 atopischen Hunden mit *Malassezia*-Dermatitis und 22 klinisch normalen Hunden zu vergleichen. Sechs verschiedenen Entdeckungssystemen wurden untersucht, um die Sensitivität zu bestimmen und nicht-spezifische Bindungen und Kreuzreaktionen zu eliminieren. Das Protokoll mit den besten Ergebnissen benutzte einen monoklonalen, gegen Hunde-IgE gerichteten Mausantikörper, einen mit alkalischer Phosphatase konjugierten Ziegenantikörper gegen Maus IgG, der dreimal durch die Säule mit Hunde IgG geschickt wurde, ein chemilumineszentes Substrat und ein digitales Bildsystem. Proteine mit einer Masse von 45, 52, 56 und 63 kDa wurden von mehr als der Hälfte der atopischen Hundeseren gebunden und repräsentierten daher wichtige Allergene. Eine Minderheit der normalen Hunde zeigte schwache IgE Bindung an diese Proteine. Die Resultate deuten daraufhin, dass die Mehrheit der atopischen Hunde mit *Malassezia*-Dermatitis im Vergleich zu normalen Hunden eine erhöhte IgE Immunantwort zeigen und dass eine IgE-medierte Immunantwort klinisch in der Pathogenese der Erkrankung wichtig sein könnte.