Serum Antibodies to Malassezia Yeasts in Canine Atopic Dermatitis

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Summary

A significant number of humans with atopic dermatitis develop Malassezia specific IgE. Immediate skin test reactivity to Malassezia has been demonstrated in atopic dogs. The aim of this study was to compare the serum IgG and IgE response to Malassezia in atopic dogs with and without clinical evidence of Malassezia dermatitis and/or otitis, non-atopic dogs with clinical evidence of Malassezia dermatitis and/or otitis and healthy dogs. Cytology was used to diagnose clinically significant Malassezia dermatitis and otitis. Contact plate cultures confirmed the validity of this technique. Reproducible enzyme-linked immunosorbant assays for Malassezia specific IgG and IgE in canine serum were established. Atopic dogs had significantly higher serum IgG and IgE levels than either healthy dogs or non-atopic dogs with clinical evidence of Malassezia dermatitis and/or otitis. There was no significant difference in IgG and IgE levels between atopic dogs with and without clinical evidence of Malassezia dermatitis and/or otitis. The implications of these findings in the pathogenesis and management of canine atopic dermatitis are discussed.

Keywords

Canine; Atopic dermatitis; Malassezia; ELISA, IgG; IgE
Introduction

Malassezia are a genus of commensal yeasts commonly found on mammalian and avian skin. Seven species are now recognised: the lipid dependent forms *M. fufur*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta* and *M. sloofiae*, and the non-lipid dependent form *M. pachydermatis*. *M. fufur*, the type species in humans, is commonly found on the skin of adolescents and young adults, where it can cause seborrhoeic and inflammatory dermatoses such as pityrosporum veriscolor and seborrhoeic dermatitis.

A significant proportion of human patients with atopic dermatitis develop a hypersensitivity response to cutaneous Malassezia. High levels of serum specific IgE found in patients with atopic dermatitis were not found in sera from either healthy patients or non-atopic patients with *Malassezia* associated diseases. Immediate and late phase skin reactivity, and lymphocyte proliferation in response to *Malassezia* extracts was also demonstrated in atopic but not non-atopic patients. A number of allergens have been identified, but the major IgE binding components appear to be 9, 20 and 96kD proteins and high molecular weight mannans.

*Malassezia* dermatitis and otitis are well recognised in canine dermatology and frequently complicate atopic dermatitis. There is growing evidence that *Malassezia* can influence the immune system in dogs. Injections of a crude *Malassezia* extract induced specific IgG and IgM titres in dogs and elevated *Malassezia* specific IgG titres have been demonstrated in Basset hounds with *Malassezia* dermatitis. Intradermal test reactivity to a crude *Malassezia* extract was seen in atopic, but not healthy, dogs. Atopic dogs with *Malassezia* dermatitis also developed larger wheals than those without. The aim of this study was to evaluate the serum IgG and IgE response to *Malassezia* in atopic and healthy dogs.

Materials and Methods
Study Populations

Dogs with atopic dermatitis were recruited from the dermatology clinic at the University of Edinburgh Small Animal Clinic. Diagnosis was based on compatible history and clinical signs, and exclusion of other causes of pruritus\(^{19-21}\). Coat brushings, skin scrapes and trial therapy were used to eliminate ectoparasites. All dogs underwent a six-week, home cooked diet trial to eliminate food intolerance. No anti-inflammatory medication was given for at least three weeks prior to examination.

All dogs with a clinical diagnosis of atopic dermatitis were intradermally skin tested with 57 allergens as follows. Dogs were sedated with 0.15 mg/kg xylazine IM. Hair was clipped from the lateral flank and 0.05ml of each allergen extract, 1/100,000 w/v histamine (positive control) and saline with phenol diluent (negative control) were injected intradermally. Test sites were assessed after 20 minutes and scored from 0 to 4 compared to the controls. Reactions >2 were considered positive\(^{21}\).

Control samples were taken from healthy dogs presented for euthanasia at the University of Edinburgh Small Animal Clinic with no history and clinical signs of pruritus or conditions likely to alter immune function for a minimum of three weeks prior to euthanasia.

Five dogs with clinically significant *Malassezia* dermatitis and/or otitis (see below) and a confirmed primary diagnosis (*Sarcoptes*, demodicosis, hypothyroidism and two with hyperadrenocorticism) were also recruited from the clinic.

*Malassezia cytology and culture*

Clinically significant *Malassezia* dermatitis and otitis was diagnosed by microscopic observation of elevated cutaneous *Malassezia* populations\(^{22-24}\). Tape-strips were collected from the groin, axillae and interdigital web of each dog. Samples from the external ear canal
were transferred to glass microscope slides by swab and heat fixed. The samples were stained using Diff-Quik® (Dade AG; Düdingen, Switzerland) and examined. *Malassezia* dermatitis and/or otitis was diagnosed if there were greater than five organisms per x400 field at any site. *Malassezia* samples were also collected using contact plates from the ear, groin, axilla, ventral lip and interdigital web of dogs with atopic dermatitis, with (n=8) and without cytological evidence of *Malassezia* dermatitis and/or otitis (n=10), non-atopic dogs with *Malassezia* overgrowth (n=2) and healthy dogs (n=7). These were cultured for 3 days at 37°C/5% CO₂ and the number of colonies counted (confluent growth was counted as 100). Colonies were re-suspended and washed in phosphate buffered saline (PBS). The specificity of each preparation was checked using Diff-Quik® stained smears, before storage at -20°C in 50% glycerol/PBS.

**Extraction of Malassezia Antigens**

The *Malassezia* suspensions were thawed, pooled, washed three times in PBS, then re-suspended in 10ml of extraction buffer (125mmol/L NH₄HCO₃, 20mmol/L e-amino-n-caproic acid, 5mmol/L ethylene-diamine-tetra-acetic acid and 1mmol/L phenyl-methane-sulphonyl-flouride) and 10ml of 0.5mm glass beads. This suspension was homogenised using a vortex mixer (Whirlimixer®; Fison, Loughborough, UK) for 10 minutes then centrifuged at 800g for 5 minutes. The supernatant was collected and centrifuged at 800g for a further 5 minutes. The protein concentration of the final supernatant was determined by spectrophotometry at 280nm and adjusted to 5.0mg/ml in PBS. The extracts were stored at -20°C. Sterile agar extracts were also prepared as negative controls.

**Sample Collection**
Sample aliquots were drawn from blood collected by jugular venepuncture from dogs with atopic dermatitis, with (n=16) and without *Malassezia* overgrowth (n=22), and non-atopic dogs with *Malassezia* overgrowth (n=5). Blood samples from healthy dogs (n=19) were collected by cardiac puncture immediately after euthanasia. Serum was separated by centrifugation and stored at -20°C.

**Enzyme Linked Immunosorbant Assay (ELISA)**

Ninety-six well ELISA plates (Dynatech, Guernsey, Channel Islands) were coated overnight at 4°C with 100µl of *Malassezia* or agar extract diluted 1/100 in coating buffer (0.1M NaHCO₃/0.1M Na₂CO₃, pH 9.6), then blocked with 150µl of 0.5% human serum albumin (HSA) in PBS/0.05% tween 20 (PBS-T) at 37°C for two hours. Appropriate wells were incubated with 50ml serum dilutions for one hour at 37°C, then 50µl of either a V200 dilution of alkaline-phosphatase conjugated polyclonal rabbit anti-canine IgG (raised by immunising rabbits against canine IgG and subsequently rendered specific for the isotype specific fc portion of the antibody by passing through a column specific for canine fab fragments, which are not isotype specific) for one hour or a V50 dilution of anti-IgE for two hours at 37°C. Plates were then incubated at 37°C with 50µl substrate solution (BCIP-NBT tablets; Sigma, Poole, UK) for either 30 minutes (IgG) or one hour (IgE) and the absorbance read at 405nm (Dynatech MR7000 plate reader). Plates were washed three times with PBS-T between each step. Reagents were diluted in 0.1%HSA/PBS-T. Blanks and control wells with antigen, serum or conjugate only, and agar were included. Each assay was performed in triplicate.

**Analysis of Results**

The optical densities (OD) were calculated from: (mean OD of the three test wells - blank OD)
minus (mean OD of three control wells without serum - blank OD). After ensuring data were
normally distributed, analyses of variance with Tukey's post-tests were used to compare the
ELISA results and colony counts between groups (Prism® and Instat®, Graphpad Inc., San
Diego, USA). Significance was set at p<0.05.
Results

Validation of the ELISAs

Standard curves were established using doubling dilutions of a single atopic and healthy serum sample (see figures 1 and 2). A positive control standard curve and negative control wells were included in each subsequent assay. No appreciable binding was seen using antigen, serum or conjugate alone, or to agar. Prior incubation of serum with antigen inhibited the assay in a dose dependant fashion. Incubation of the positive control serum at 56°C for one hour abolished binding in the IgE, but not IgG, assay. The mean intra-assay variations calculated from the controls were 4.1% and 6.2% for the IgG and IgE ELISA respectively. The mean interassay variations were 7.7% and 9.1% respectively. Serum was diluted $\frac{1}{40}$ (IgG) and $\frac{1}{20}$ (IgE) in the following assays.

ELISAs for Malassezia-specific IgG and IgE

Atopic dogs had significantly higher levels of anti-Malassezia IgG and IgE than non-atopic dogs ($p<0.05$) (see table 1, figures 3 and 4). There was no significant difference between atopic dogs with and without clinical evidence of Malassezia dermatitis and/or otitis, nor between non-atopic dogs with clinical evidence of Malassezia dermatitis and/or otitis and healthy dogs.

Malassezia Colony Counts (see table 2)

The mean colony counts were significantly higher at all sites except the lower lip in atopic dogs with clinical evidence of Malassezia dermatitis and/or otitis (i.e. elevated numbers of Malassezia seen on cytology) than in either atopic dogs without Malassezia dermatitis or otitis and healthy dogs ($p>0.05$). There was no significant difference in the colony counts at
any site between the latter two groups. Because of the small sample size, data from the non-atopic dogs with *Malassezia* overgrowth was not included in the analysis. *Malassezia* were isolated from at least one site in 24 of the 27 dogs sampled.


**Discussion**

The atopic dogs in this study had higher levels of both IgG and IgE than non-atopic dogs, irrespective of whether or not they had clinical evidence of *Malassezia* dermatitis and/or otitis at the time of sampling. Previous investigations have shown that most dogs harbour commensal *Malassezia* populations, particularly in the ear and at mucosal sites. Despite this exposure, there was only a limited antibody response in healthy animals in this study, possibly because *Malassezia* are normally outwith the body compartment. The high antibody levels in atopic dogs with *Malassezia* dermatitis and otitis could be part of an ongoing inflammatory response. However, this was not mirrored in the non-atopic dogs with *Malassezia* overgrowth, although the group size was small and 4/5 dogs had primary conditions that can be immunosuppressive. Another study found that Bassets with *Malassezia* dermatitis did mount an IgG response, but it did not appear to be protective.

Equally high levels of IgG and IgE were found in atopic dogs with and without clinical evidence of *Malassezia* dermatitis and/or otitis. This is in contrast to earlier findings where atopic dogs with *Malassezia* dermatitis had larger skin test wheals than those without. This may reflect differences in cutaneous and circulating levels IgE levels. It is possible that some atopic dogs have high circulating but low cutaneous mast cell bound *Malassezia* specific IgE levels. Skin tests may therefore be a more accurate reflection of sensitisation and better able to distinguish dogs with active inflammation. In this study, cases without clinical evidence of *Malassezia* dermatitis and/or otitis at the time of sampling had no immediate history of *Malassezia* dermatitis or otitis, but this does not necessarily rule out prior infection. It is possible that IgG and IgE titres are maintained for long periods after resolution, or that commensal populations are a sufficient reservoir of antigen for ongoing stimulation.

Atopic dogs may also be more exposed to *Malassezia* antigens than healthy animals. Licking
might seed organisms from mucosal to cutaneous sites, where increased temperature, moisture, inflammation and trauma may facilitate their exposure to the immune system\textsuperscript{14,15}. Ineffective barrier function in atopic skin may also facilitate the absorption of antigen and a subsequent immune response.

Atopic individuals could also be skewed towards a humoral immune response to cutaneous organisms. TC-type responses involving IL-4, IL-5, IL-6 and IL-13 support humoral immune reactions, including IgE production, whereas T\textsubscript{H2}-type responses involving IL-2 and IFN\textgreek{g} promote cell mediated responses and inhibit IgE production\textsuperscript{30}. Under this hypothesis, healthy individuals would have a T\textsubscript{H1} dominant response, which is capable of clearing fungal infections but is otherwise down regulated to avoid type IV hypersensitivity\textsuperscript{31}. Atopic individuals would have an unregulated TV dominant response with high IgG and IgE titres\textsuperscript{30}. Evidence of this has been found in human patients with atopic dermatitis where Malassezia reactive T-cells had a Tm-type skewed cytokine pattern\textsuperscript{30,32}. Tin-type responses have recently been detected in atopic canine skin (T.J.Nuttall, J.R.Lamb, P.A.Knight, S.M.McAleese and P.B.Hill, submitted for publication), raising the possibility that this skewing also occurs in canine atopic dermatitis.

Seventy-four per cent of the atopic dogs in this study had Malassezia specific IgE levels greater than the upper 99\% confidence limit for healthy dogs. This agrees with previous findings where immediate skin test reactivity present in the majority of atopic dogs was not seen in any healthy dogs\textsuperscript{18}. Together, these data suggest a proportion of atopic dogs could be sensitised to Malassezia antigens, which might contribute to the burden of ongoing atopic disease. This has important implications for the management of these cases, which could require long-term antifungal treatment, even in the absence of elevated cutaneous populations or clinical Malassezia dermatitis and/or otitis. In human patients results from topical therapy with ketoconazole have been disappointing, possibly because treatment failed to eliminate all
cutaneous and mucosal *Malassezia*\(^{33}\). Despite this, systemic therapy has been effective in cases of atopic dermatitis associated with *Malassezia* sensitivity in humans\(^8\). Specific immunotherapy in dogs sensitised to *Malassezia* antigens is another alternative. One report suggested this was effective in a case of *Malassezia* dermatitis in a dog\(^{16}\).

Microbial inhibition is also a possibility. No facilities for identification of the *Malassezia* isolated were available in this study. The morphological characteristics of the colonies and yeasts were most compatible with *M. pachydermatis*, but the extracts used could have included a range of antigens from different *Malassezia* species or strains of *M. pachydermatis*\(^{2,34}\). Gel separation and immunoblotting could identify major and minor allergens, and whether these are unique to certain *M. pachydermatis* strains or are common allergens. In the former case it might be possible to colonise an animal with a non-allergenic *Malassezia* strain. Antigenic differences have been identified in different *M. pachydermatis* strains\(^{35}\), although cross-reactivity is common across *Malassezia* strains and other fungi, such as *Candida*\(^{33}\).

This study has validated an ELISA for *Malassezia* specific IgG and IgE in dogs. The lack of binding in control wells and with heated serum, antigen inhibition and low intra- and interassay variation all demonstrate the specificity and reliability of the assay\(^{27}\). Cross reaction with environmental fungi was unlikely as no skin test reactivity to *Altenaria*, *Penicillium*, *Botrytis* or oat, wheat and barley smut was seen.

Tape-strip and otic swab cytology are routinely in the author’s clinic to diagnose elevated *Malassezia* numbers associated with dermatitis and otitis. *Malassezia* colony counts were significantly greater in dogs with cytological evidence of elevated *Malassezia* numbers than in those without. This agrees with previous findings that cytological assessment is a valid technique to diagnose *Malassezia* dermatitis and otitis in a clinical setting\(^{22-24}\). Dogs were therefore grouped into those with and without clinical evidence of *Malassezia* dermatitis.
and/or otitis on the basis of cytological data.

In conclusion, this study confirms that atopic dogs have higher levels of *Malassezia* specific IgG and IgE than healthy dogs, which are not dependant on the presence of elevated numbers of *Malassezia*. Further work is needed to identify major and minor allergens, and to study the efficacy of topical or systemic treatment and immunotherapy.
Acknowledgements

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References


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an enzyme-linked immunosorbent assay containing monoclonal murine anti-canine IgE. 


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Figure 1 - Standard Curves for IgG ELISA
Figure 2 - Standard curve for IgE ELISA
Table 1 - ODs for IgG and IgE binding to *Malassezia* extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (sd) OD at 405nm x1000</th>
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<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>Atopic dogs (n=22)</td>
<td>374 (+/-145)</td>
</tr>
<tr>
<td>Atopic dogs with <em>Malassezia</em> overgrowth (n=16)</td>
<td>390 (+/-129)</td>
</tr>
<tr>
<td>Non-atopic dogs with <em>Malassezia</em> overgrowth (n=5)</td>
<td>168 (+/-48)</td>
</tr>
<tr>
<td>Healthy dogs (n=19)</td>
<td>177 (+/-97)</td>
</tr>
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Table 2 - Mean (sd) *Malassezia* colony counts/contact plate

<table>
<thead>
<tr>
<th>Sample</th>
<th>Axilla</th>
<th>Groin</th>
<th>Interdigital web</th>
<th>Lower lip</th>
<th>Ear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atopic dogs (n=10)</td>
<td>0.5 (+/-0.7)</td>
<td>0.7 (+/-1.2)</td>
<td>3.2 (+/-3.6)</td>
<td>2.8 (+/-3.4)</td>
<td>9.8 (+/-12.3)</td>
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<tr>
<td>Atopic dogs with <em>Malassezia</em> overgrowth (n=8)</td>
<td>35.1 (+/-28.2)</td>
<td>14.3 (+/-13.5)</td>
<td>27.3 (+/-23.0)</td>
<td>3.4 (+/-5.5)</td>
<td>45.8 (+/-37.2)</td>
</tr>
<tr>
<td>Non-atopic dogs with <em>Malassezia</em> overgrowth (n=2)</td>
<td>60.5 (+/-177.2)</td>
<td>5.5 (+/-6.0)</td>
<td>13.5 (+/-5.6)</td>
<td>0.5 (+/-0.7)</td>
<td>4.5 (+/-6.0)</td>
</tr>
<tr>
<td>Healthy dogs (n=7)</td>
<td>0</td>
<td>0</td>
<td>0.14 (+/-0.4)</td>
<td>15.8 (+/-37.2)</td>
<td>0.57 (+/-0.8)</td>
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