

Serum antibodies to *Malassezia* yeasts in canine atopic dermatitis

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Abstract Significant numbers 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, nonatopic 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 immunosorbent 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 nonatopic 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: atopic dermatitis, canine, ELISA, IgE, IgG, *Malassezia*.

INTRODUCTION

Malassezia are a genus of commensal yeasts commonly found on mammalian and avian skin.¹ Seven species are now recognized: the lipid-dependent forms *M. fufur*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta* and *M. sloofiae*, and the nonlipid-dependent form *M. pachydermatis*.² *Malassezia 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 versicolor and seborrhoeic dermatitis.^{1,3}

A significant proportion of human patients with atopic dermatitis develop a hypersensitivity response to cutaneous *Malassezia*.^{4–6} High levels of serum-specific IgE found in patients with atopic dermatitis were not found in sera from either healthy patients or nonatopic patients with *Malassezia*-associated diseases.⁷ Immediate and late-phase skin reactivity, and lymphocyte proliferation in response to *Malassezia* extracts were also demonstrated in atopic but not nonatopic patients.^{4,7–11} A number of allergens has been identified, but the major IgE-binding components appear to be 9-, 20- and 96-kDa proteins and high molecular mass mannans.¹²

Malassezia dermatitis and otitis are well-recognized in canine dermatology and frequently complicate atopic dermatitis.^{13–15} There is growing evidence that *Malassezia* can influence the immune system in dogs. Injec-

tions of a crude *Malassezia* extract induced specific IgG and IgM titres in dogs,¹⁶ and elevated *Malassezia*-specific IgG titres have been demonstrated in Basset Hounds with *Malassezia* dermatitis.¹⁷ Intradermal test reactivity to a crude *Malassezia* extract was seen in atopic, but not healthy, dogs.¹⁸ 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 6-week, home-cooked diet trial to eliminate food intolerance. No anti-inflammatory medication was given for at least 3 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⁻¹ xylazine intramuscularly (IM). Hair was clipped from the lateral flank and 0.05 mL of each allergen extract, 0.1 mg/ml histamine (positive control) and saline with phenol diluent (negative control) were injected intradermally. Test sites were assessed after 20 min and scored from

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0 to 4 compared with 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 3 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 were 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, Düringen, Switzerland) and examined. *Malassezia* dermatitis and/or otitis was diagnosed if there were > 5 organisms per ×400 field at any site.

Malassezia samples were also collected using contact plates²⁵ 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 ($n = 10$), nonatopic 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 resuspended 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

Malassezia suspensions were thawed, pooled, washed three times in PBS, then resuspended in 10 mL of extraction buffer (125 mmol L^{–1} NH₄HCO₃, 20 mmol L^{–1} σ -amino-*n*-caproic acid, 5 mmol L^{–1} ethylene-diamine-tetra-acetic acid and 1 mmol L^{–1} phenyl-methane-sulphonyl-flouride) and 10 mL of 0.5-mm glass beads. This suspension was homogenized using a vortex mixer (Whirlimixer®; Fison, Loughborough, UK) for 10 min then centrifuged at 800 g for 5 min. The supernatant was collected and centrifuged at 800 g for a further 5 min. The protein concentration of the final supernatant was determined by spectrophotometry at 280 nm and adjusted to 5.0 mg mL^{–1} 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 ($n = 22$) *Malassezia* overgrowth, and nonatopic 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

Ninety-six well enzyme-linked immunosorbant assay (ELISA) plates (Dynatech, Guernsey, Channel Islands) were coated overnight at 4 °C with 100 μ L of *Malassezia* or agar extract diluted 1:100 in coating buffer (0.1 M NaHCO₃/0.1 M Na₂CO₃, pH 9.6), then blocked with 150 μ L of 0.5% human serum albumin (HSA) in PBS/0.05% Tween 20 (PBS-T) at 37 °C for 2 h. Appropriate wells were incubated with 50 μ L serum dilutions for 1 h at 37 °C, then 50 μ L of either a 1:200 dilution of alkaline phosphatase-conjugated polyclonal rabbit anticanine IgG (raised by immunizing 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 1 hour or a 1:50 dilution of anti-IgE²⁶ for 2 h at 37 °C. Plates were then incubated at 37 °C with 50 μ L substrate solution (BCIP-NBT tablets; Sigma, Poole, UK) for either 30 min (IgG) or 1 h (IgE) and the absorbance read at 405 nm (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

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 Fig. 1a and b). 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 1 h 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 1:40 (IgG) and 1:20 (IgE) in the following assays.

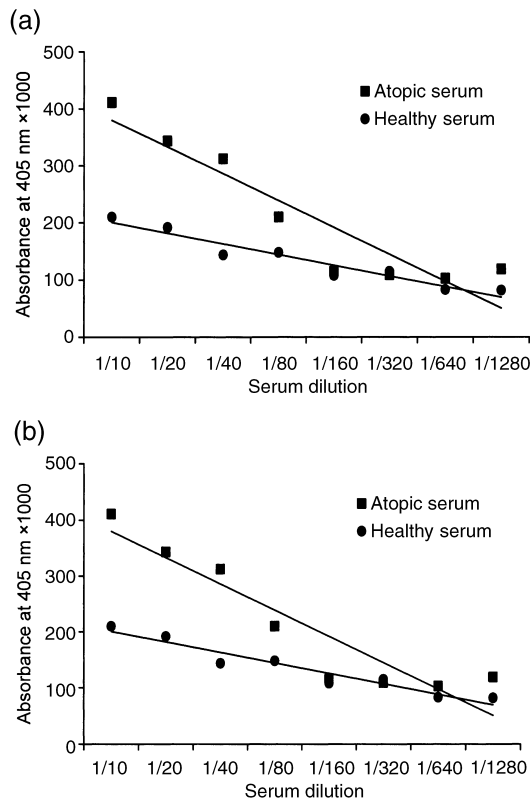


Figure 1. Standard curves established for the *Malassezia*-specific IgG(a) and IgE(b) ELISAs using doubling dilutions of single positive control atopic and negative control healthy sera.

ELISAs for Malassezia-specific IgG and IgE

Atopic dogs had significantly higher levels of anti-*Malassezia* IgG and IgE than nonatopic dogs ($P < 0.05$) (Table 1, Fig. 2a and b). There was no significant

Table 1. Optical density (OD) values for IgG and IgE binding to *Malassezia* extracts

Sample	Mean (SD) OD at 405 nm×1000	
	IgG	IgE
Atopic dogs ($n = 22$)	374 (± 145)	344 (± 103)
Atopic dogs with <i>Malassezia</i> overgrowth ($n = 16$)	390 (± 129)	368 (± 145)
Non-atopic dogs with <i>Malassezia</i> overgrowth ($n = 5$)	168 (± 48)	163 (± 75)
Healthy dogs ($n = 19$)	177 (± 97)	165 (± 70)

difference between atopic dogs with and without clinical evidence of *Malassezia* dermatitis and/or otitis, or between nonatopic dogs with clinical evidence of *Malassezia* dermatitis and/or otitis and healthy dogs.

Malassezia colony counts

The mean colony counts (Table 2) 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 nonatopic dogs with *Malassezia* overgrowth were not included in the analysis. *Malassezia* were isolated from at least one site in 24 of the 27 dogs sampled.

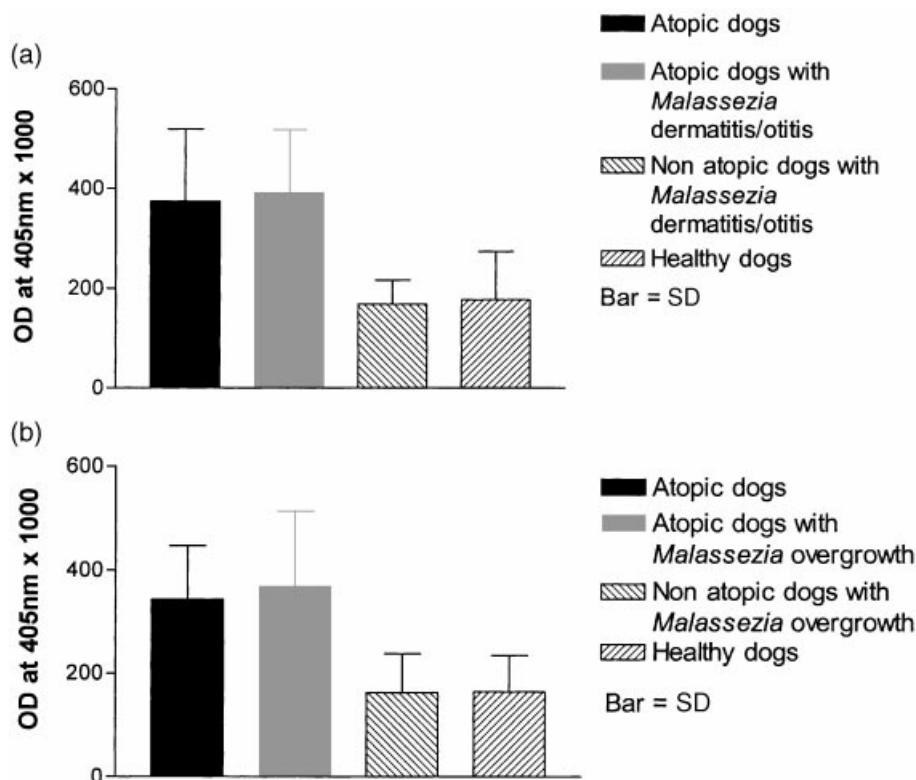


Figure 2. Mean optical density measured in *Malassezia*-specific IgG(a) and IgE(b) ELISAs using atopic and non atopic sera.

Table 2. Mean (SD) *Malassezia* colony counts/contact plate

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

DISCUSSION

The atopic dogs in this study had higher levels of both IgG and IgE than nonatopic dogs, irrespective of whether 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.^{27,28} 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 nonatopic dogs with *Malassezia* overgrowth, although the group size was small and four of the five dogs had primary conditions that can be immunosuppressive. Another study found that Basset Hounds with *Malassezia* dermatitis did mount an IgG response, but it did not appear to be protective.¹⁷

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 in which atopic dogs with *Malassezia* dermatitis had larger skin test wheals than those without.¹⁸ 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 sensitization 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.^{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. T-helper type 2 (Th2)-type responses involving interleukin (IL)-4, IL-5, IL-6 and IL-13 support humoral

immune reactions, including IgE production, whereas Th1-type responses involving IL-2 and interferon (IFN) γ promote cell-mediated responses and inhibit IgE production.²⁹ Under this hypothesis, healthy individuals would have a Th1-dominant response, which is capable of clearing fungal infections but is otherwise downregulated to avoid type IV hypersensitivity.³⁰ Atopic individuals would have an unregulated Th2-dominant response with high IgG and IgE titres.²⁹ Evidence of this has been found in human patients with atopic dermatitis in whom *Malassezia* reactive T cells had a Th2-type skewed cytokine pattern.^{29,31} Th2-type responses have recently been detected in atopic canine skin (T.J. Nuttall, J.R. Lamb, P.A. Knight *et al.*, unpublished observations), 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 in which immediate skin-test reactivity present in the majority of atopic dogs was not seen in any healthy dogs.¹⁸ Together, these data suggest that a proportion of atopic dogs could be sensitized 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*.³² Despite this, systemic therapy has been effective in cases of atopic dermatitis associated with *Malassezia* sensitivity in humans.⁸ Specific immunotherapy in dogs sensitized to *Malassezia* antigens is another alternative. One report suggested that 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*.^{2,33} 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 colonize an animal with a nonallergenic *Malassezia* strain. Antigenic differences have been identified in

different *M. pachydermatis* strains,³⁴ although cross-reactivity is common across *Malassezia* strains and other fungi, such as *Candida*.³²

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.³⁵ Cross-reaction with environmental fungi was unlikely as no skin test reactivity to *Alternaria*, *Penicillium*, *Botrytis* or oat, wheat and barley smut was seen.

Tape-strip and otic swab cytology are used 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 those 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.

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Résumé Dans l'espèce humaine, un nombre significatif de patients souffrant de dermatite atopique développent des IgE spécifiques vis à vis de *Malassezia*. Une réaction immédiate à *Malassezia* a été démontrée par test cutané chez les chiens atopiques. Le but de cette étude était de comparer la réponse sérique en IgG et IgE vis à vis de *Malassezia* chez des chiens atopiques avec ou sans évidence de dermatite et/ou otite à *Malassezia*, chez des chiens non atopiques présentant une dermatite et/ou une otite à *Malassezia* et chez des chiens sains. Les dermatites et otites à *Malassezia* ont été diagnostiquées par examen cytologique. Des cultures sur boîtes de contact ont permis de confirmer la validité de cette technique. Des tests ELISA reproductibles pour la mesure des IgG et des IgE spécifiques de *Malassezia* ont été établis. Les chiens atopiques présentaient des taux sériques d'IgG et d'IgE significativement plus élevés que les chiens sains ou que les chiens non atopiques souffrant de dermatite et/ou d'otite à *Malassezia*. Il n'a pas été observé de différence entre les taux d'IgG ou d'IgE pour les chiens atopiques avec ou sans signe clinique de dermatite et/ou d'otite à *Malassezia*. Les implications de ces observations dans la pathogénie et le traitement de la dermatite atopique chez le chien sont discutées.

Resumen Un número significativo de humanos con dermatitis atópica desarrollan IgE *Malassezia*-específicas. Se ha demostrado reactividad en la prueba cutánea a *Malassezia* en perros atópicos. El objetivo de este estudio fue comparar las respuestas de IgG y IgE séricas a *Malassezia* en perros atópicos con y sin evidencia clínica de dermatitis por *Malassezia* y/o otitis y perros sanos. La citología fue usada para diagnosticar la dermatitis y otitis por *Malassezia* clínicamente significativa. Los cultivos en placa por contacto confirmaron la validez de esta técnica. Se establecieron pruebas reproducibles de inmunoabsorción ligada a enzimas para IgG e IgE *Malassezia*-específicas en suero canino. Los perros atópicos mostraron niveles de IgG e IgE séricos significativamente más elevados que en perros sanos o en perros no atópicos con signos clínicos de dermatitis y/o otitis por *Malassezia*. No existía diferencia significativa entre los niveles de IgG e IgE entre perros atópicos con y sin evidencia clínica de dermatitis y/o otitis por *Malassezia*. Se discuten las implicaciones de estos hallazgos en la patogenesis y manejo de la dermatitis atópica canina.

Zusammenfassung Eine signifikante Anzahl von Menschen mit atopischer Dermatitis produzieren *Malassezia*-spezifische IgE Antikörper. Überempfindlichkeit vom Soforttyp wurde bei atopischen Hunden nachgewiesen. Das Ziel dieser Studie war der Vergleich von Serum IgG und IgE Antikörpern gegen *Malassezia* bei atopischen Hunden mit und ohne klinischen Anzeichen der *Malassezia*-Dermatitis und/oder Otitis, nichtatopischen Hunden mit klinischen Anzeichen der *Malassezia*-Dermatitis und/oder Otitis und gesunden Hunden. Klinisch signifikante *Malassezia* Dermatitis und Otitis wurde zytologisch nachgewiesen. Kontaktplattenkulturen bestätigten die Gültigkeit dieser Methode. Reproduzierbare Enzym-linked Immunoassays für *Malassezia*-spezifische IgG und IgE Antikörper wurden entwickelt. Atopische Hunde hatten signifikant höhere Serum IgG und IgE Konzentrationen als gesunde Hunde und als nonatopische Hunde mit klinischen Anzeichen von *Malassezia*-Dermatitis und/oder Otitis. Zwischen den IgG und IgE Konzentrationen von atopischen Hunden mit und jenen von atopischen Hunden ohne klinische Anzeichen von *Malassezia*-Dermatitis und Otitis bestand kein signifikanter Unterschied. Die Implikationen dieser Befunde für die Pathogenese und das Management von atopischer Dermatitis beim Hund wird diskutiert.