

Humoral measurement of type-1 hypersensitivity reactions to a commercial *Malassezia* allergen

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(Received 12 January 2005; accepted 17 May 2005)

Abstract *Malassezia pachydermatis* is considered to be a contributing factor to canine atopic dermatitis (AD). The purpose of this study was to investigate the humoral response to a commercially produced *M. pachydermatis* extract. Fifteen atopic dogs with *Malassezia* overgrowth on the skin (MD), 16 atopic dogs without MD, three atopic dogs with overgrowth of *Malassezia* in the ears only (MO), and 12 normal dogs were intradermally tested with *M. pachydermatis* extract at 50, 100, 250, 500, 1000, 2000 and 4000 PNU mL⁻¹. All dogs were evaluated cytologically by cutaneous tape strip and bilateral ear exudate sampling to determine presence of MD or MO. Each had serum evaluated for anti-*Malassezia* IgE using three *Malassezia* extracts with an ELISA assay. The irritant threshold concentration at which healthy nonatopic dogs ceased to react was 1000 PNU mL⁻¹. There was a significant difference in intradermal test reactivity between the atopic groups. At this dilution, 93% (14/15) of the atopic MD group, 31% (5/16) of the atopic group without MD or MO, and 100% (3/3) of the atopic MO only group reacted. There were no significant differences in the serum IgE levels as measured by the Greer ELISA assay, between any groups using any of the three extracts. These results support that Greer's *M. pachydermatis* extract is useful for intradermal testing of dogs with an allergic phenotype, and that atopics with MD are more likely to have a type-1 *Malassezia* hypersensitivity than those without. The ELISA assay may require further development in order to be useful for the diagnosis of *Malassezia* hypersensitivity.

INTRODUCTION

Malassezia pachydermatis is a nonlipid-dependent lipophilic yeast which may be isolated from normal canine skin in low numbers. However, when there are changes in the microenvironment and natural defence mechanisms of the epidermis, increased colonization by *M. pachydermatis* may occur.^{1–3} The number of organisms that may be demonstrated on the skin of healthy dogs during cytological analysis is variable among individual dogs and depends on sampling site and breed. Normal basset hounds have been shown to have higher carriage of *Malassezia* than other dogs.⁴ In a previous report, it was demonstrated that < 3 yeast/1.6 cm² body surface area is normal in 70% of normal dogs and < 10 yeast/1.6 cm² is normal in 95% of normal dogs⁵ (Fig. 1). When yeast numbers beyond this figure are identified on atopic dogs with active dermatitis, there is cytological and clinical evidence of *Malassezia* dermatitis. These dogs predictably respond to anti-fungal therapy. Cases of *Malassezia*-associated dermatitis typically present with marked inflammation and pruritus, and previous studies have demonstrated that atopic dogs with *Malassezia* overgrowth can mount cell-mediated

and humoral (immediate; type I) hypersensitivity reactions to crude extracts of *M. pachydermatis*.^{6–10}

Peripheral blood mononuclear cells isolated from atopic dogs with *Malassezia* overgrowth exhibited significantly increased blastogenic responses to *M. pachydermatis* extracts compared to those isolated from normal dogs.⁷ Evidence for immediate hypersensitivity includes increased intradermal test (IDT) reactivity to crude extracts of *M. pachydermatis*, increased serum concentrations of anti-*Malassezia* IgE as determined by the use of ELISA, and the successful passive transfer of cutaneous anaphylaxis via atopic canine serum (which harboured high titres of anti-*Malassezia* IgE) to normal dogs.^{6,8,9} Demonstration of passive transfer by the Prausnitz–Kustner test confirms the functionality of canine anti-*Malassezia* IgE.⁹ In addition, western immunoblotting of sera from dogs with *Malassezia* overgrowth has identified several candidate allergens produced by *M. pachydermatis* that are recognized by atopic dogs.¹⁰

Malassezia-associated dermatitis and otitis are common clinical problems and are often exacerbated in conjunction with atopic flares.¹³ Clinical identification of atopic dogs with type I hypersensitivity reactions to a commercially available and standardized *M. pachydermatis* extract would be beneficial, as it could assist in choosing dogs that might be candidates for modification of the dysregulated immune response by allergen-specific immunotherapy. Both IDT and ELISA techniques are used in clinical practice. The clinical identification of hypersensitivity requires that a commercial allergenic

This study was presented at the annual North American Veterinary Dermatology Forum in Sarasota, FL, USA, April 2005.
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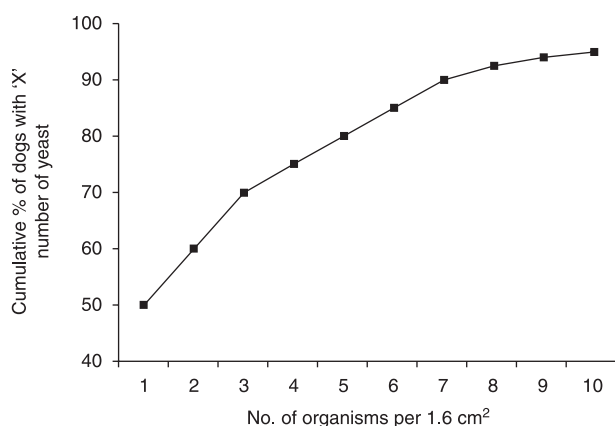


Figure 1. Graph of *Malassezia* yeast population density on normal canine skin, showing the cumulative percentage of normal dogs exhibiting less than 'X' number of yeast on direct impression smear, as described by Kennis *et al.*⁵ Yeast counts were limited to 1.6 cm² area of a glass slide, with microscopic examination performed at $\times 100$ under oil immersion. Impressions were taken from two adjacent interdigital webs of a forepaw, the dorsal aspect of a forelimb, one axillary fossa, the ventrum and dorsum of the neck, the chin, perioral region, inguinal region, and umbilical region. The slide with the greatest number of yeast was chosen from each dog for graphic analysis. Figure reprinted with permission from an article by Morris *et al.*⁸

extract be used for IDT at the optimal concentration for detecting true sensitization. The optimal concentration of an allergen is the highest concentration that fails to produce a reaction in 90% of normal individuals (the 'irritant' threshold), but that is capable of identifying a significant number of clinically allergic individuals (the 'allergy detection' threshold).^{11,12} Testing with an allergen at a concentration above the threshold may result in a nonspecific false positive ('irritant') reaction.

The purposes of this study were to evaluate the canine humoral response to a new USDA-approved commercial extract of *M. pachydermatis* in healthy dogs without an overgrowth of *Malassezia* and in atopic dogs with and without an overgrowth of *M. pachydermatis* via IDT and an ELISA technique, and to determine the optimal (threshold) concentration of the allergenic extract for IDT.

MATERIALS AND METHODS

Animals

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Twelve privately owned, clinically normal dogs were tested for reactivity to a commercial *M. pachydermatis* extract (see below for specifics), and serum was collected for an anti-*M. pachydermatis* IgE ELISA assay. Inclusion criteria for normal subjects included the following: normal dermatological examination with no history of pruritus, skin or ear disease, and lack of cytological evidence of excessive *Malassezia* colonization of the skin and ear canals. For these purposes, excessive colonization of the skin was defined as ≥ 10 *Malassezia* organisms per 1.6 cm² area on a microscopic

glass slide containing an acetate tape preparation of the skin. Samples were obtained from four anatomical locations: the interdigital space of a forepaw, the ventral area of the neck, an axillary fossa, and the inguinal region.⁵ The acetate tape preparation was performed by gently clipping the area with a no. 40 blade and gentle removal of the hair with a dry gauze sponge. The tape was then pressed firmly against the skin two times in succession, labelled, and stained by modified Wright's stain (Diff-Quik®, Dade AG, Düringen, Switzerland), for subsequent microscope evaluation.¹³ Ear cytology was obtained by a cotton swab of each external ear canal, at the transition between the vertical and horizontal canal. The swab specimens were then rolled on to a glass microscope slide, labelled, heat fixed and stained by modified Wright's stain (Diff-Quik®, Dade AG), for subsequent microscope evaluation. Excessive colonization of the ear canals was defined as ≥ 5 yeast organism per $\times 100$ oil immersion field of exudate from each ear canal.^{14,15} Dogs were also excluded if they had received steroidal medications orally, parenterally, or topically within the last 30 days, injectable steroids within 3 months, antihistamines within 14 days, or if they had been bathed within the last 14 days. The 12 normal dogs included eight spayed females and four castrated males. Their ages ranged from 1 to 12 years (median age 5 years). Normal dogs were not intradermally tested for the standard panel of aeroallergens.

For the study group, 31 client-owned dogs were recruited from the small animal dermatology clinic at the Matthew J. Ryan Veterinary Hospital of the University of Pennsylvania. Inclusion criteria for the study group were a clinical diagnosis of AD based upon compatible history and clinical signs, and exclusion of other causes of pruritus.^{11,12} Coat brushings, skin scrapings and miticide trial therapies were used to rule out ectoparasitism on all dogs. Dogs with nonseasonal pruritus underwent dietary elimination trials of 8 weeks minimum duration on either home-prepared or commercial hydrolysed dog foods to rule out adverse food reaction. Withdrawal from medications and bathing was as described for normal dogs (see above). Dogs suspected of AD were tested intradermally with a standard panel of 62 commercial aero- and insect allergens. Dogs were sedated with 750 mg m⁻² medetomidine (Dormitor; Pfizer Animal Health, New York, NY, USA), and hair was clipped from the lateral hemithorax. Histamine 1 : 100 000 w/v (positive control), phenol-buffered saline (negative control) and each allergen were injected intradermally at a volume of 0.05 mL. Test sites were evaluated for a wheal and flare response subjectively on the basis of erythema, induration, turgidity, and surface area at 15 and 30 min postinjection. Reactions were graded on a scale of 0–4+ (0 = no reaction compared to the saline control; 4+ = the maximum reaction compared to a histamine phosphate control). Wheal and flare reactions $\geq 2+$ were considered clinically relevant. Only the dogs with at least three positive reactions to the commercial aeroallergens on the intradermal skin test were included in the atopic group.

Allergen

M. pachydermatis (Strain AD 92643, established from a clinical isolate obtained from the ear canal of an atopic dog) was grown in liquid trypticase medium (Greer laboratories, Lenoir, NC, USA) for 17 days at 25–30 °C. The identity of the isolate was based on the following microscopic and biochemical profile: globose to ellipsoidal yeast-like conidia that are round at one end and blunt at the other. From the blunt end, bottle-shaped, unipolar budding occurs from the phialides with small collarettes. Biochemical tests (API20C, bioMerieux Vitek, Hazelwood, MO, USA) showed an assimilation reaction profile consistent with *Malassezia* sp. Fatty acids were not required for growth.

The culture filtrate was separated from the cells by filtration, concentrated and dialysed by ultrafiltration using an Amicon H10P5 hollow fibre and lyophilized. The cellular fraction, recovered by filtration was further processed by washing in acetone and Ball-milled to prepare a powder for extraction. The acetone-powdered cells were extracted at 1 : 10 w/v in 0.1 M ammonium bicarbonate pH 7.8 for 18 h at 2–8 °C. The extract was centrifuged at 10 000 g for 30 min, and the supernatant recovered and dialysed for 48 h in a 6000 MW cut-off Spectra/Por dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA, USA) against 0.01 M ammonium bicarbonate pH 7.8 at 2–8 °C. The resulting extract was clarified by filtration and lyophilized. Extracts used for skin testing were prepared by extracting the acetone cell powder at 1 : 10 w/v in Coca's extraction fluid (5 g L⁻¹ NaCl; 5.4 g L⁻¹ NaHCO₃; 0.4% v/v phenol) for 18 h at 2–8 °C. The extract was clarified by centrifugation and sterilized by filtration through a 0.2 µm Millipore membrane filter. Sterility and safety testing was performed as part of lot release testing and in conformance with Greer's USDA license for allergen extract manufacturer.

The *Malassezia pachydermatis* extract was diluted with phosphate-buffered saline to obtain the following seven test concentrations: 4000 PNU (protein nitrogen units) mL⁻¹, 2000 PNU mL⁻¹, 1000 PNU mL⁻¹, 500 PNU mL⁻¹, 250 PNU mL⁻¹, 100 PNU mL⁻¹ and 50 PNU mL⁻¹ for intradermal testing. The protein nitrogen unit (PNU) of the extracts was determined using the Kjeldahl method after precipitation of protein using phosphotungstic acid. The diluted allergens were stored in glass vials and new dilutions were made every 2 weeks.

Intradermal testing

For both normal and atopic dogs, 0.05 mL of each of the dilutions of allergen (4000 PNU mL⁻¹, 2000 PNU mL⁻¹, 1000 PNU mL⁻¹, 500 PNU mL⁻¹, 250 PNU mL⁻¹, 100 PNU mL⁻¹ and 50 PNU mL⁻¹) was injected, along with the positive control (1 : 100 000 w/v histamine), and the negative control (phenol-buffered saline). The 31 dogs with AD were tested immediately after their standard IDT. Test sites were evaluated subjectively by the same investigator for a wheal and flare response and based upon erythema, induration, turgidity, and

surface area after 15 and 30 min postinjection. Evaluation of reactions utilized the same scale as for the standard panel of aeroallergens.

Cytology

All atopic dogs were evaluated for cytological evidence of *M. pachydermatis* after IDT evaluation was completed. Acetate tape preparations were collected by the standard technique from at least four of the following anatomical locations: the most erythematous interdigital space, ventral neck fold, axillary fossa, inguinal region, periocular, perineal, and perioral regions.^{5,13} Samples from both external ear canals were transferred to glass microscope slides by swab and heat fixed. The samples were stained using the modified Wright's stain (Diff-Quik®, Dade AG), and examined at ×100 objective under oil immersion. Dogs with clinically significant *Malassezia* overgrowth of the skin (MD) and ears (MO), respectively, were diagnosed when at least one slide was positive for yeast as defined by ≥ 10 yeast organisms/1.6 cm² (MD) or ≥ 5 yeast organisms on average per ×100 oil immersion field (MO).¹⁴ Absence of MD and MO were determined by the same cytological criteria used for the clinically normal dogs.

The atopic dogs were then grouped on the basis of results of the IDT and cytological evaluation into three groups: atopic dogs with MD (MD+), atopic dogs with only MO (MD–MO+), and atopic dogs with neither MD nor MO (atopic control dogs) (MD–MO–).

Sera collection

Five ml of blood was collected by jugular venepuncture from all dogs and sera were separated by centrifugation and stored at –80 °C until used for ELISA assay.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) used throughout this study is a noncompetitive, solid-phase enzyme immunoassay that incorporates a mixture of biotinylated monoclonal anticanine IgE antibodies as the primary tracer for allergen-specific IgE molecules. Polystyrene microtitre wells (Immulon 4HBX, Thermo Laboratory systems, Franklin, MA, USA) were coated overnight at 4 °C with either the *Malassezia* cellular, culture filtrate, or the Greer skin test preparation, each at a concentration previously shown in preliminary experiments to saturate the wells. These determined extract concentrations were as follows: 1 : 500 dilution of 1 mg mL⁻¹ cellular extract preparations, 1 : 300 dilution of 3 mg mL⁻¹ culture filtrate preparation, and 1 : 100 dilution of 20 000 PNU mL⁻¹ of the Greer skin test preparation.

Preliminary data not shown here verified the following ELISA conditions: ELISA plate selection (Immulon 4 was chosen out of the following compared plastics: 1B, 2B, 4B Immulon and NUNO0), the coating condition with each extract, the anti IgE dilution factor, incubation time, and the sera dilution factor.

Plate wells without allergen served as background controls; positive and negative control serum directed

towards June grass pollen allergens served as calibrators to normalize the ELISA results.

No true positive reference sample exists for *Malassezia* antigen ELISA. But a pooled sera was used to simulate a positive reference sample. This reference sample included sera from five of the atopic cases with *Malassezia*-associated dermatitis (MD+) who reacted with a positive wheal and flare response of ≥ 2 to all dilution concentrations tested (4000 PNU mL⁻¹, 2000 PNU mL⁻¹, 1000 PNU mL⁻¹, 500 PNU mL⁻¹, 250 PNU mL⁻¹, 100 PNU mL⁻¹, and 50 PNU mL⁻¹).

Unreacted sites were blocked with a 1% solution of neutral monoethanolamine. For evaluation, 100 μ L of 3-fold serial dilutions of each serum sample in 50 mM TRIS buffered saline pH 7.5 was added to duplicate wells. Following an overnight incubation at 4 °C in a humidified chamber, the wells were washed for four cycles with TRIS-buffered saline containing 0.05% Tween 20. Biotinylated anti-IgE (Greer Laboratories, Lenoir NC) (100 μ L), diluted in TRIS buffered saline pH 7.5, was added to each well and incubation was continued for 2 h at room temperature (22 °C). Unreacted anti-IgE-biotin was removed by washing and 100 μ L of streptavidin alkaline phosphatase was added to each well. Following a 1-h incubation period the wells were washed ($\times 4$) as described above, and 100 μ L of p-ntiroyphenyl phosphate (pNPP) substrate was added to each well. Substrate development was allowed to continue for precisely 1 h. The reaction was stopped by adding 50 μ L of 20 mM cysteine to each well. The absorbance of each well was measured at 405 nm using a Molecular Device (Sunnyvale, CA, USA) Versamax tunable microplate reader. The absorbance of each well on the plate was normalized on the basis of the calibrators. Each individual serum background control value was then subtracted from the results for each sample. Finally, the two duplicate wells were averaged. This net result was the reported mean adjusted absorbance value.

Statistical analysis

To determine differences between the atopic dogs in the group without MD (MD-) and with MD (MD+) for intradermal wheal and flare response ≥ 2 at each dilution, the Fisher's exact test was used. To determine differences between MD- and MD+ for the single sera ELISA results, the Student's *t*-test was used. All analyses were performed using SAS statistical software (Version 9.1, SAS Institute, Cary, NC, USA). A *P*-value of 0.05 was considered statistically significant. There were not enough animals in the other two groups to make a statistical comparison. Only MD- and MD+ groups were compared.

RESULTS

All dogs in all groups reacted to the positive control with a significant reaction. None of the dogs in any of the groups reacted significantly to the negative control.

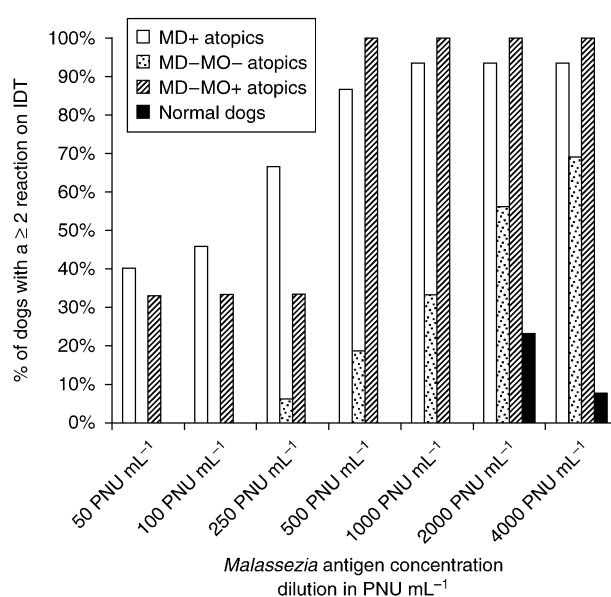


Figure 2. Percentage of atopic and normal dogs in each of the four groups with a positive reaction of ≥ 2 on IDT at each *Malassezia* dilution concentration.

In the group of 12 healthy nonatopic dogs, at the concentration of 4000 PNU mL⁻¹ four dogs reacted with scores ≥ 2 . At the concentration of 2000 PNU mL⁻¹ three of the dogs reacted with scores ≥ 2 . None of the dogs reacted at the lower concentrations of 1000 PNU mL⁻¹, 500 PNU mL⁻¹, 250 PNU mL⁻¹, 100 PNU mL⁻¹, and 50 PNU mL⁻¹. Therefore, the optimal (threshold) concentration was 1000 PNU mL⁻¹. This is because it is the highest concentration where greater than 90% of normal dogs failed to react. This was chosen as the concentration for use in evaluating differences between atopic dogs with and without MD.

Cytology results divided the 34 atopic dogs into the following groups. There were 15 dogs that had an overgrowth of *Malassezia* on their skin (MD+). Four of the 15 dogs in this MD+ group also had an overgrowth of *Malassezia* in their external canal. There were 16 dogs that did not have an overgrowth of yeast on their skin or in their ears. (MD-MO-). Finally, there were three dogs that did not have MD but had MO (MD-MO+). This atopic group (MD-MO+) was too small to be compared statistically to the other two groups.

Intradermal test results demonstrated that in the MD-MO+ group, all three of the dogs reacted at the calculated threshold dilution of 1000 PNU mL⁻¹. Of the 15 atopic dogs in the MD+ group, 14 (93%) reacted at the threshold dilution of 1000 PNU mL⁻¹ whereas only five of the 16 atopic dogs without MD (MD-MO-) (31%) reacted. This was a significant difference (*P* < 0.001, Fisher's exact test). The dogs in the MD+ group also had significantly stronger wheal and flare reactions than the MD-MO- group at less concentrated dilutions (Fig. 2). Of the 4/15 dogs in the MD+ group that also had an overgrowth of *Malassezia* in the ears (subgroup MD+MO+), all four of these dogs reacted with ≥ 2 response to the threshold concentration.

Table 1. Mean adjusted absorbance values, as detected by ELISA, for anti-*Malassezia* IgE in canine sera

ELISA variables	Group	Median	Mean	SD	Max. value	Min. value	# of cases
Culture filtrate sera diluted 1 : 10	MD+ atopics	106	166	155	644	31	15
	MD–MO– atopics	93	166	287	1270	31	16
	Normal	139	149	60	258	66	12
Culture filtrate sera diluted 1 : 30	MD+ atopics	56	121	180	734	0	15
	MD–MO– atopics	64	119	208	914	16	16
	Normal	65	73	34	133	23	12
Cellular extract sera diluted 1 : 10	MD+ atopics	121	208	208	831	0	15
	MD–MO– atopics	82	183	328	1418	0	16
	Normal	109	112	84	312	15	12
Cellular extract sera diluted 1 : 30	MD+ atopics	60	144	205	795	0	15
	MD–MO– atopics	55	132	224	960	0	16
	Normal	55	73	42	145	30	12
Greer extract sera diluted 1 : 10	MD+ atopics	167	206	164	706	38	15
	MD–MO– atopics	98	178	234	1039	38	16
	Normal	132	152	80	296	58	12
Greer extract sera diluted 1 : 30	MD+ atopics	86	148	188	754	0	15
	MD–MO– atopics	60	128	220	954	12	16
	Normal	61	72	43	151	18	12

Upon further historical evaluation of the five cases in the MD–MO– group that reacted with a positive response to the threshold concentration (31%), it was determined that one had a known episode of MD 7 months prior to admission in the study, while another one of these had an episode of MO 4 months prior to study admission.

The ELISA absorbance values were determined for sera of all dogs. No significant differences were detected between the groups, at either serum dilution or when using any of the three different allergen extracts for antibody capture (Table 1).

DISCUSSION

The 'irritant' threshold is the highest concentration of an allergen that does not produce a positive result in > 90% of normal individuals.¹¹ None of the normal dogs in this study reacted at 1000 PNU mL⁻¹. However, higher concentrations induced a mean positive wheal and flare reaction of ≥ 2 in more than 10% of the normal dogs, confirming 1000 PNU mL⁻¹ to be the 'irritant' threshold. Previous studies have evaluated the 'irritant' threshold concentrations of crude extracts of *M. pachydermatis*. One study found it to be 2 µg mL⁻¹ for a lyophilized *M. pachydermatis* extract and most of its fractions,⁸ while another found it to be 2000 µg mL⁻¹.⁹ This illustrates the need for a standardized commercial allergen, as these studies were performed by the same investigator.

The commercial extract used in this study uses the PNU mL⁻¹ assay to standardize the concentration batch to batch. This assay has been adopted by the FDA and USDA for labelling of allergenic extracts. The assay has a repeatability coefficient of variation of 5% and a reproducibility coefficient of variation of circa 14%. Therefore, it has reasonable repeatability.

When evaluating an extract for its utility in true allergy detection, the 'irritant' threshold (evaluated in

normal dogs) is used as a reference point for evaluating allergic dogs. In this study, 93% of the MD+ atopic dogs and 31% of MD–MO– atopic dogs were reactive at the 'irritant' threshold, which demonstrates that the 'irritant' and 'allergy detection' thresholds are similar or the same.

There was a strong statistical difference when comparing the two atopic groups. The MD+ dogs had greater IDT responses than did MD–MO– dogs ($P = 0.00063$). There was also a significant difference between these two groups at several other dilutions. The proposed mechanism by which atopic dogs sensitize to *Malassezia* yeast is epicutaneous contact with allergen produced by the organism, which induces the atopic cascade. A likely contributing factor to sensitization by atopic dogs is impairment of stratum corneum barrier function due to inflammation and self-trauma. Dogs with increased numbers of yeast on the skin would be expected to have a greater chance of sensitization. However, 31% of the MD– group did react on IDT. There are several possible explanations for this finding (as reported in a previous study).⁸

The use of a culture technique has been shown to be more sensitive than cytological tape staining in identifying the presence of *Malassezia* on the skin.¹⁵ Therefore, if this test had been employed instead of the cytological tape analysis, it is possible these MD– but IDT+ dogs would have been assigned to the MD+ group, while the rest of the MD– group would still have been assigned to the appropriate group. However, it is known that *Malassezia* is a commensal organism on normal skin, and it is therefore possible that this technique would have raised the amount of false positives assigned to the MD+ group. Also, to the authors' knowledge, no studies have compared type I hypersensitivity reactions between atopics with and without culture positive *Malassezia*, whereas cytological staining has been shown to consistently find significant differences between these two groups.^{7–9}

These dogs may have been sensitized by a previous episode of MD or MO that subsequently cleared before enrolment in the study. One of the five cases in the MD–MO– group, which reacted with a positive response to the threshold concentration, had a known episode of MD 7 months prior to admission in the study, while another case in this group had an episode of MO 4 months prior to study admission. Whether MO contributes to type I hypersensitivity in atopic dogs is as yet unknown. *Malassezia* yeast is commonly associated with otitis externa in atopic dogs.¹⁴ All of the dogs in the MD–MO+ group in this study reacted with a positive reaction to the ‘irritant’ threshold. This is in contrast to previous findings, where two dogs in an MD–MO+ group did not react upon IDT.⁷ In the previous study, it was postulated that the dogs in the MD–MO+ group did not elicit a type I hypersensitivity reaction because of the possible protective microenvironment of the ear. They postulated that high wax and lipid content in the ear perhaps interfered with recognition of the organism by the local T-cell population. The results of this study do not support this theory. However, the case numbers of this particular group in both studies are too low for statistical analysis. Elucidation of the role of *Malassezia* hypersensitivity in otitis will require further study.

In addition, this study's cytological definition of *Malassezia* overgrowth may not be sufficiently sensitive in identifying the number of organisms required for sensitization. The number of organisms used to define MD was based upon cytological analysis of healthy dogs with normal skin and may not account for the physiological and microenvironmental changes that occur in inflamed skin that could allow fewer organisms to exert a pathogenic role. It may be this group for which positive IDT is most valuable, as these dogs may be sensitized to *Malassezia* yeast but negative on cytology by our current definition. Future studies evaluating the response to antifungal therapy of MD–IDT+ dogs are indicated.

In this study, IDT showed a strong discrimination between groups whereas ELISA did not detect a difference between groups. Immediate IDT reactivity and antigen specific IgE serology both attempt to measure humoral responses to allergens. Therefore, one would expect findings to be at least correlative. However, it is not uncommon to have conflicting results for environmental aeroallergens between IDT and *in vitro* test results.^{16,17} There are several factors that could have contributed to the lack of correlation between the two tests studied here.

Concentrations of anti-*Malassezia* IgE were distributed similarly in all groups. The relatively high amounts of circulating antibody in the MD– atopics and normal dogs may have been a result of prior episodes of MD or MO that were unknown in the history and inactive at the time of study enrolment. Similarly, the relatively high/moderate IgE levels documented in the normal group may reflect a normal response seen in healthy dogs to commensal populations.¹⁸ However,

this contrasts with the results of another study which found significantly higher IgE levels in the sera of atopic dogs than in normal dogs.⁶

The differences between the two tests studied here could also be due to the heterogenicity of IgE, which has been documented in dogs. Two subforms of canine IgE have been identified. One of these subforms has been shown to be less reactive to monoclonal anti-canine IgE antibody in ELISA in comparison to a polyclonal anti-IgE antibody.¹⁹ It is possible that the monoclonal antibody we used did not recognize the same *Malassezia*-specific IgE that was responsible for *in vivo* responsiveness on IDT.

The lack of correlation between the two tests may also be due to the ELISA design and further modification may be needed before it can be offered for reliable screening of atopic patients with MD. One potential problem may be the lack of specificity caused by using crude extracts for preparing the allergosorbents. Use of selected purified *Malassezia* allergens could increase the specificity of the assay.

In conclusion, this study confirms that many atopic dogs develop type I hypersensitivity to *M. pachydermatis*, and that intradermal testing with Greer Laboratories' *M. pachydermatis* extract may be useful in clinical evaluation of these patients. Future studies should focus on enhancing the ELISA's capabilities and in identifying the major allergens within this extract that are recognized by atopic dogs with *Malassezia* hypersensitivity. Studies of yeast cross-reactivity with environmental fungi should also be considered.

ACKNOWLEDGEMENT

Greer Laboratories, Lenoir, NC, USA provided the *Malassezia* allergen for IDT, and the laboratory equipment for the ELISA evaluation.

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Résumé *Malassezia pachydermatis* est considérée comme un facteur contribuant aux lésions de dermatite atopique canine (AD). Le but de cet essai était d'étudier la réponse humorale à un extrait commercial de *M. pachydermatis*. Quinze chiens atopiques avec une prolifération de *Malassezia* sur la peau (MD), 16 chiens atopiques sans MD, trois chiens atopiques avec prolifération de *Malassezia* dans les oreilles seulement (MO), et 12 chiens normaux ont été testés par voie intradermique avec un extrait de *M. pachydermatis* (Greer laboratories, Lenoir, NC) à 50, 100, 250, 500, 1000, 2000, et 4000 PNU/ml. Tous les chiens ont été évalués cytologiquement par test à la cellophane adhésive et écouvillonnage auriculaire pour déterminer la présence de MD ou MO. Un dosage des IgE anti-*Malassezia* a été réalisé dans tous les cas en utilisant trois extraits de *Malassezia* par test ELISA. La concentration irritante des extraits à laquelle tous les chiens sains non atopiques ne réagissaient plus était de 1000 PNU/ml. Une différence significative de réactivité intradermique a été observée entre les groupes de chiens atopiques. A cette dilution, 93% (14/15) des chiens atopiques du groupe MD, 31% (5/16) des chiens du groupe atopique sans MD ou MO, et 100% (3/3) des chiens atopiques à MO ont réagi. Aucune différence significative pour les taux d'IgE sériques par ELISA n'a été observée. Ces résultats suggèrent que l'extrait de *M. pachydermatis* commercialisé par Greer est utile pour tester par voie intradermique les chiens à phénotype allergique, et que les chiens atopiques à MD sont plus à risque d'avoir une réaction d'hypersensibilité de type I à *Malassezia* que les chiens sans MD. Le test ELISA en revanche nécessite des études supplémentaires pour être utile dans le diagnostic des hypersensibilités à *Malassezia*.

Resumen Se considera que *Malassezia pachydermatis* es un factor que contribuye a producir dermatitis atópica (AD). El objetivo de este estudio fue investigar la respuesta humoral a extracto comercial de *M. pachydermatis*. Quince perros atópicos con sobrecrecimiento de *Malassezia* en la piel (MD), 16 perros atópicos sin MD, tres perros atópicos con sobrecrecimiento de *Malassezia* sólo en los oídos (MO), y 12 perros normales fueron probados vía intradérmica con extracto de *M. Pachydermatis* (Laboratorios Greer, Lenoir, North Carolina, USA) a concentraciones de 50, 100, 250, 500, 1000, 2000 y 4000 PNU/ml. Todos los perros se evaluaron mediante citología de piel obtenida con cinta adhesiva y mediante muestreo bilateral del exudado de los oídos para determinar la presencia de MD o MO. Se evaluó el suero de los perros para la presencia de IgE frente a *Malassezia* utilizando tres extractos de *Malassezia* en un ensayo de ELISA. El nivel de irritante al cual los perros sanos no atópicos dejaron de reaccionar fue 1000 PNU/ml. Se encontró una diferencia significativa en la reacción intradérmica entre los diferentes grupos de perros atópicos. A esta dilución, 93% (14/15) de los perros atópicos con MD, 31% (5/16) de los perros atópicos sin MD ni MO, y 100% (3/3) de los perros atópicos sólo con MO reaccionaron al extracto. No observamos diferencias significativas en los niveles de IgE en el suero entre los distintos grupos, valorados mediante el ensayo de ELISA de laboratorios Greer utilizando cualquiera de los tres extractos. Estos resultados indican que el extracto de *M. Pachydermatis* de los laboratorios Greer es útil para la prueba intradérmica de los perros con fenotipo alérgico, y que es más probable que los perros alérgicos con MD presenten hipersensibilidad

de tipo I frente a *Malassezia* que los perros sin MD. El ensayo de ELISA posiblemente requiera una mejor caracterización para ser útil en el diagnóstico de hipersensibilidad frente a *Malassezia*.

Zusammenfassung *Malassezia pachydermatis* wird als beitragender Faktor zur caninen atopischen Dermatitis betrachtet. Das Ziel dieser Studie war die Untersuchung der humoralen Antwort auf einen kommerziell produzierten *M. pachydermatis* Extrakt. Fünfzehn Hunde mit übermäßigem Wachstum von Malassezien auf der Haut (MD), 16 atopische Hunde ohne MD, 3 atopische Hunde mit übermäßigem Wachstum von Malassezien nur in den Ohren (MO), und 12 normale Hunde wurden mit *M. pachydermatis* Extrakt (Greer Laboratories, Lenoir, NC) mit Konzentrationen von 50, 100, 250, 500, 1000, 2000, und 4000 PNU/ml intradermal getestet. Alle Hunde wurden auch zytologisch untersucht mittels Klebestreifenabklatsch und Probenahme des Exsudates aus beiden Ohren, um das Vorhandensein von MD oder MO zu ermitteln. Bei jedem Hund wurde Serum auf anti-Malassezia IgE untersucht mittels dreier Malassezien Extrakte in einem ELISA Test. Die reizende Grenzwertkonzentration, bei der gesunde nicht atopische Hunde aufhörten zu reagieren, lag bei 1000 PNU/ml. Es bestand ein signifikanter Unterschied bei den Reaktionen im Intradermaltest zwischen den atopischen Gruppen. Bei dieser Verdünnung reagierten 93% (14/15) der atopischen MD Gruppe, 31% (5/16) der atopischen Gruppe ohne MD oder MO, und 100% (3/3) der atopischen MO Gruppe. Zwischen den verschiedenen Gruppen bestand kein signifikanter Unterschied im Bezug auf Serum IgE Werte, die mittels Greer ELISA Test unter Verwendung der drei unterschiedlichen Extrakte gemessen wurden. Diese Ergebnisse bestätigen, dass Greer's *M. pachydermatis* Extrakt nützlich ist für das intradermale Testen von Hunden mit einem allergischen Phänotyp und dass Atopiker mit MD häufiger eine Malassezien Hypersensibilität vom Typ-1 aufweisen als solche ohne. Um für die Diagnose einer Malassezien Überempfindlichkeit nützlich zu sein, bedarf es möglicherweise einer Weiterentwicklung des ELISA Tests.