

Use of an amplified ELISA technique for detection of a house dust mite allergen (*Der f 1*) in skin and coat dust samples from dogs

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Objective—To use an amplified ELISA technique to document the presence and quantify the concentration of the house dust mite allergen, *Der f 1*, in skin and coat dust samples collected from dogs.

Animals—29 pet dogs of various breeds.

Procedure—Dogs were weighed, and body surface area in square meters was determined. Skin and coat dust samples were obtained by vacuuming dogs. Collected dust was analyzed by use of standard and amplified ELISA techniques.

Results—By use of the standard ELISA technique, *Der f 1* was detected in skin and coat dust samples from 6 of 29 (21%) dogs. Mean concentration of *Der f 1* in the 6 samples with positive assay results was 16.16 ng/mL (range, 5.61 to 31.24 ng/mL). Samples with negative assay results were retested for dust mite allergen by use of an amplified ELISA technique; an additional 14 dogs had positive assay results. Mean concentration of allergen was 0.36 ng/mL (range, 0.19 to 2.20 ng/mL). Combining both techniques, 20 of 29 (69%) dogs had positive assay results for *Der f 1*.

Conclusions and Clinical Relevance—Results of our study indicate that house dust mite allergens are present on the skin and in the coat of dogs, and this source of allergen may act as a reservoir for allergen exposure in hypersensitive dogs. Use of an amplified ELISA technique to determine environmental concentrations of house dust mite allergens in homes and on dogs will help to identify the relationship between immunologic findings and environmental exposures in dogs with atopic dermatitis. (*Am J Vet Res* 2003;64:162–165)

Atopic dermatitis is defined as a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features and is associated most commonly with IgE antibodies to environmental allergens.¹ The house dust mites *Dermatophagoides farinae* and *D pteronyssinus* are amongst the most common and important allergens in dogs with atopic dermatitis.^{2,3} At the veterinary teaching hospital of The Ohio State University, > 68% of

dogs with atopic dermatitis have hypersensitivity to *D farinae*.⁴ In humans, sensitivity to dust mites is frequently observed in patients with bronchial asthma and allergic rhinitis.⁵ Sensitization to dust mites occurs in approximately 51% of children with atopic dermatitis and 60% of adult asthmatics.^{6,7} Substantial risk for the development of asthma in atopic humans has been proposed from exposure to concentrations of > 10 µg of *Dermatophagoides* group-1 allergens/g of dust.⁵

The amount of exposure to mites and allergens depends on the concentration or quantity of allergen in reservoirs in or around homes. Generally accepted techniques for estimating the amount of exposure to dust mite allergens include mite counts, assays of specific mite allergens, and measurement of mite fecal matter.⁸ Immunoassays are sensitive, specific, and can produce quantitative results for a given allergen in absolute units. Monoclonal antibodies for detection of mite allergens (groups 1 and 2) are commercially available. Allergens are generally measured from samples collected by vacuuming reservoirs of settled dust within homes, such as the carpet, bedding, and upholstered furniture. Allergen quantification may be limited by the ability to collect sufficient amounts of dust, extraction protocol, and sensitivity of immunochemical methods used. In analysis of *Dermatophagoides* group-1 allergens in small samples obtained from air sample collection, allergen concentrations are generally below the detection limit of 0.2 µg/g or 10 ng/mL for the standard ELISA.⁹ Airborne mite allergen concentrations are commonly about 30 pg/m³.¹⁰ Recently, an amplified ELISA technique was developed for *Dermatophagoides* group-2 allergens to overcome this obstacle.¹¹ The amplified technique resulted in a 15-fold increase in the sensitivity of the standard ELISA for *Dermatophagoides* group-2 allergens, thereby lowering the detection limit to 300 pg/mL. These results indicated for the first time that a substantial portion (20.6%) of *Der p 2* was associated with small particles that were in the respirable range (1.1 to 4.7 µm). In this technique, an enzyme cycling system was used to amplify the colorimetric signal generated by an alkaline phosphatase label. Two enzymes, diaphorase and alcohol dehydrogenase, interconvert NADH into NAD with each turn of the cycle, generating a colored formazan molecule.

The human scalp has been reported as a reservoir for house dust mites,¹² and the house dust mite allergen *Der p 1* has been detected in human hair dust.¹³ We hypothesized that house dust mite allergens are present on the skin and in the coat of dogs, and this source

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of allergen may act as a reservoir for allergen exposure in hypersensitive dogs. We sought to document the presence and quantify the concentration of the house dust mite allergen, *Der f 1*, in skin and coat dust samples collected from dogs. Further, we sought to perform the amplified ELISA technique to quantify *Der f 1* concentrations that were below the detection limit of the standard ELISA.

Materials and Methods

Animals—Dust samples from the skin and coat were collected for our study from 2 groups of dogs. Group-A dogs were pets belonging to staff and students of the College of Veterinary Medicine, The Ohio State University, whereas group-B dogs were pets brought to a private veterinary practice for boarding or grooming. Selection of dogs for inclusion in our study was made irrespective of medical problem and individual characteristics (eg, breed, age, and sex).

Detailed questionnaires on the clinical background and history were completed for group-A dogs. The questionnaires were designed to obtain data on insecticides used on pets and in the home, frequency of dog bathing, the location and characteristics of the dogs' resting areas, and historical features of any chronic skin or ear disease. A diagnosis of atopic dermatitis in group-A dogs was made according to accepted criteria,¹⁴ which included a compatible history, typical clinical features, and diagnostic testing to rule out differential diagnoses such as flea allergy dermatitis, sarcoptic acariasis, cutaneous adverse food reactions, bacterial dermatitis, and *Malassezia* dermatitis. Dogs with atopic dermatitis had positive reactions to intradermal testing, performed according to standard procedure,¹⁵ or had positive allergen-specific IgE serologic test^a results to allergens that were suspected or known to be in their environment and that were consistent with the nature of the disease in the individual patient. Group-B dogs were tested for allergen content in skin and coat dust samples, and no information on clinical history and background was obtained. In addition, group-B dogs were not examined for atopic dermatitis.

Dust sample collection technique—Dogs were weighed, and body surface area in square meters was determined by use of the following standard equation:¹⁶

$$m^2 = (10.1 \times \text{body weight in grams}^{2/3}) \times 10^{-4}$$

On the basis of the standardized protocol of vacuuming 1 m² for 2 minutes for collection of samples of dust for allergen identification and quantification from environmental areas,¹⁷⁻¹⁸ we calculated the time of vacuuming each dog according to calculated body surface area. A dog with 1 m² of body surface area was vacuumed for 2 minutes, and dogs with < 1 m² of body surface area were vacuumed for 12 seconds for each 0.1 m² of body surface area. The entire body was vacuumed. Some dogs were lightly sedated with xylazine hydrochloride (1.0 mg/kg, IV) and atropine sulfate (0.02 mg/kg, IV) to facilitate sample collection.

Skin and coat dust samples were collected by using a vacuum cleaner^b with a special dust trap. Samples were stored in sealed plastic bags at 4°C until the *Der f 1* ELISA was performed. The vacuum cleaner head and tubing distal to the filter were thoroughly cleaned and scrubbed in boiling water between sample collections.

Standard *Der f 1* ELISA—House dust mite allergen (ie, *Der f 1*) concentrations were determined by use of a standard ELISA technique.⁹ All collected samples were sieved through a 355- μ m-diameter pore filter to remove hair. One hundred milligrams of each sample was weighed and extracted in 2.0

mL of PBS-Tween 20 solution overnight at 4°C. For samples that weighed < 50 mg, 1.0 mL of PBS-Tween 20 solution was added. For samples that weighed between 50 to 100 mg, the appropriate amount of buffer was added to make a sample dilution of 50 mg/mL. To perform a standard *Der f 1* ELISA, 96 well microtiter plates^c were coated with *Der f 1* specific monoclonal antibodies^d (200 ng/well) in 50mM sodium carbonate-sodium bicarbonate buffer (pH, 9.6) at 4°C overnight. Microtiter plates were then incubated with 100 μ L of 1% bovine serum albumin in PBS-Tween 20 solution for 1 hour. Next, 100 μ L of doubling serial dilutions from 250 to 0.5 ng/mL of reference *Der f 1*^e or 1:10 to 1:80 dilutions of skin and coat dust samples were added in duplicate wells and incubated for 1 hour. Three negative controls consisted of duplicate wells that lacked 1 of the following: primary antibody, secondary antibody, or *Der f 1*. Following incubation, wells were incubated with 100 μ L of a 1:1000 dilution of biotinylated anti-*Der f 1* monoclonal antibodies^f and allowed to incubate for 1 hour. Wells were then washed 3 times and coated with 100 μ L of a 1:1000 dilution of streptavidin-peroxidase solution and allowed to incubate for 30 minutes. Bound biotinylated-labeled antibody was detected by adding 100 μ L of 1mM 2', 2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) with the addition of 1 μ L of H₂O₂/mL of solution. The reaction was stopped after 5 minutes with 100 mL of sodium azide and the optical density read by use of a microplate spectrophotometer^g at 405 nm. Concentration of *Der f 1* allergen was determined by interpolation from reference curves obtained by the use of an immunoassay software program^h and reported as nanograms of *Der f 1*/mL of sample. Allergen standards^e used had interassay coefficients of variation typically under 15%.

Amplified *Der f 1* ELISA—An amplified ELISA was performed by use of an amplification kitⁱ on samples that had negative results for *Der f 1* on the standard ELISA. Microtiter plates^c were coated with *Der f 1* specific monoclonal antibody^d (200 ng/well) overnight at 4°C. Plates were washed 3 times and incubated in 1% bovine serum albumin in wash buffer^j for 1 hour at room temperature (approx 22°C). The wash buffer in this technique differed from the standard ELISA wash buffer and consisted of a proprietary tris-buffered solutionⁱ with detergent containing sodium azide (15 mmol/L) as a preservative. After an additional 3 washes, plates were incubated for 1 hour with 100 μ L of doubling serial dilutions from 5 ng/mL to 0.01 ng/mL of reference *Der f 1*^e or 1:10 and 1:20 dilutions of skin and coat dust samples. Three negative controls consisted of duplicate wells that lacked 1 of the following: primary antibody, secondary antibody, or *Der f 1*. Plates were washed 5 times and incubated for 1 hour with 100 μ L of a 1:1,000 dilution of biotinylated anti-*Der f 1* monoclonal antibodies. Dilutions were made with wash buffer^j instead of PBS-Tween 20 solution that contained 1% bovine serum albumin as in the standard ELISA. Phosphate buffer saline solution is known to inhibit the conjugate enzyme used in the amplification kitⁱ and affect sensitivity. Wells were then washed 3 times, coated with 100 μ L of a 1:1,000 dilution of streptavidin-alkaline phosphatase, and allowed to incubate for 1 hour. A final 5 washes were performed and then the amplification reagents were used. One hundred microliters of amplification reagent Aⁱ followed by 100 μ L of amplification reagent Bⁱ were added to each well. Plates were developed and read when the first standard reached an optical density of 2.7 at 490 nm. Concentration of *Der f 1* was determined and reported as previously described.

Data analysis—Regression analysis was performed to determine the correlation between recovered dust and the amount of *Der f 1* present in samples. A value of $P < 0.05$ was considered significant.

Results

Samples were collected from a total of 29 dogs, with 16 group-A dogs for which historical and clinical data were available and 13 group-B dogs. Mean weight for all samples collected from dogs was 37.42 mg with a range of 14 to 75 mg. One group-B dog had < 10 mg of recoverable dust collected and was excluded from our study, resulting in 12 group-B dogs. In group-A dogs, 11 of 16 had short hair. Of the 16 group-A dogs, 6 were a mixed breed and 4 were Greyhounds. Only 1 group-A dog spent a substantial amount of time outdoors. Most group-A dogs spent most of their time indoors on dog beds (5/16), on the floor (4/16), or on the couch (4/16).

By use of the standard ELISA technique, *Der f 1* was detected in the skin and coat dust samples from 6 of 29 (21%) dogs (Fig 1). The mean concentration of *Der f 1* in the 6 samples with positive assay results was 16.16 ng/mL with a range of 5.61 to 31.24 ng/mL. Samples with negative assay results were then retested for dust mite allergen by use of the amplified technique. An additional 14 dogs had positive assay results. The mean concentration of allergen was 0.36 ng/mL with a range of 0.19 to 2.20 ng/mL. Combining both techniques, 20 of 29 (69%) dogs had positive assay results for *Der f 1*. Regression analysis revealed no correlation ($r^2 = 0.009$, $P = 0.68$) between the amount of dust collected and the concentration of quantifiable *Der f 1*.

Comparison of the historical and clinical data among group-A dogs, with and without detectable *Der f 1* in their skin and coat dust samples, did not reveal any differences between each subgroup. Routine use of insecticides on dogs for flea control was performed on 11 of 12 group-A dogs with positive assay results and all 4 group-A dogs with negative assay results. Fipronil was the insecticide commonly used (12/16 dogs). In addition, the mean time since the last bathing prior to sample collection was 3 weeks in group-A dogs with positive assay results and 5.3 weeks in group-A dogs with negative assay results. Seven of 13 group-A dogs

that had *Der f 1* detected in skin and coat dust samples had atopic dermatitis. However, only 1 group-A dog had house dust mite hypersensitivity. Thus, no association between the concentration of *Der f 1* in skin and coat dust samples and atopic disease was found.

Discussion

To our knowledge, this report is the first to document house dust mite allergen found skin and coat dust samples collected from dogs. It has recently been reported that the major allergen of *D farinae* for dogs is *Der f 15*¹⁹ and that *Der f 1* is not a major allergen in dogs.^{20,21} As such, it may be more important to examine skin and coat dust samples from dogs for *Der f 15*. However, detection reagents for this allergen are not commercially available. Despite the fact that *Der f 1* is not the major allergen for dogs, we believe it is still relevant to quantify this allergen as a marker for mite exposure.

Because of the lower detection limit achieved with the amplified assay, the potential of the skin and coat of dogs serving as a reservoir for exposure to house dust mite allergens must be considered. Recent evidence supports an epidermal route of allergen challenge and entry in dogs with atopic dermatitis.²²⁻²⁵ Three observations support this hypothesis. First, Langerhans cells are commonly found in clusters and in greater numbers in lesional atopic skin compared to nonlesional atopic skin or skin from normal nonatopic dogs. Second, $\gamma\delta$ -T cells are specialized lymphocytes in mucosal and epithelial immunity that are commonly seen in lesional atopic canine skin. Lastly, intact and degranulated eosinophils are found below the stratum corneum in lesional atopic skin but not in nonlesional atopic skin of dogs. The presence of house dust mite allergens on the surface microenvironment of the skin of dogs supports hypotheses on the epidermal route of allergen entry. Concentrations of quantifiable mite allergen from skin and coat dust samples of dogs in our study were similar to mean *Der p 1* concentrations (22 ng/mL) found in hair dust samples from the scalps of humans.¹²

To our knowledge, our report is the first on the use of an amplified ELISA technique for detection of *Dermatophagoides* group-1 allergens. The increase in sensitivity achieved by use of the enzyme cycling system¹ is comparable to a previously published technique for *Dermatophagoides* group-2 allergen amplification.¹¹ Because a primary objective of our study was to develop an improved technique to measure *Dermatophagoides* group-1 allergens, no attempt was made to quantify *Dermatophagoides* group-2 allergens. Using an amplified technique, we were able to lower the detection limit for *Der f 1* to 0.01 ng/mL, which is a 50-fold improvement. Compared with the standard ELISA that uses streptavidin-peroxidase as a label, colorimetric signal amplification was achieved by use of alkaline phosphatase and an enzyme cycling system. A similar technique could be developed with the appropriate sensitivity for *Der f 15* to study exposure and sensitization in dogs. Identifying environmental allergen concentrations in homes and on dogs will help to identify the relationship between immunologic find-

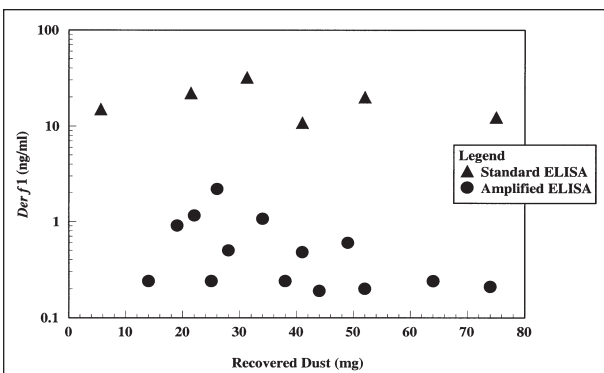


Figure 1—Concentration of house dust mite antigen (*Der f 1*) versus amount of skin and coat dust samples collected from 29 dogs by vacuuming. By use of the standard ELISA technique, *Der f 1* was detected in skin and coat dust samples from 6 dogs. Samples with negative assay results were retested for dust mite allergen by use of an amplified ELISA technique; an additional 14 dogs had positive assay results. Regression analysis revealed no correlation between concentration of allergen and the amount of dust collected on the skin and in the coat from dogs ($r^2 = 0.009$, $P = 0.68$).

ings and environmental exposures in dogs with atopic dermatitis. An improved technique with lower detection limits creates many opportunities to understand allergic diseases in human and veterinary medicine.

Understanding clinical signs and mechanisms of sensitization in humans with allergic diseases and in dogs with atopic dermatitis requires more knowledge on specific routes of allergen exposure. Results of prior study²⁶ indicate that *D. farinae* is the predominate mite species in Ohio with population densities correlated with indoor physical and climatic factors. Recent findings²⁷ suggest that the presence of a pet in homes in central Ohio does not influence the density of *Dermatophagoides* group-1 allergens in homes. The exact clinical relevance of ecological relationships between house dust mites and dogs with low concentrations of *Der f 1* on their skin and coat is unknown and should be examined in further detail. The amplified assay may provide a useful tool to experimentally test this question.

^aAllercept, Heska Corp, Fort Collins, Colo.

^bFilter Queen, HMI Industries Inc, Cleveland, Ohio.

^cImmunlon 2, Dynex Technologies, Chantilly, Va.

^dAnti *Der f 1* 6A8, Indoor Biotechnologies LTD, Charlottesville, Va.

^e*Der f 1* allergen standard ST-DF1, Indoor Biotechnologies LTD, Charlottesville, Va.

^fAnti *Der f 1* 4C1, Indoor Biotechnologies LTD, Charlottesville, Va.

^gMRX Revelation, Thermo Labsystems, Beverly, Mass.

^hStatLIA Immunoassay Software, Brendan Scientific Corp, Grosse Point Farms, Mich.

ⁱAmpliQ Kit, Dako LTD, Cambridgeshire, UK.

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