

Assessment of cross-reactivity among five species of house dust and storage mites

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What is known about the topic of this paper

- House dust mites and, possibly, storage mites are important sources of environmental allergens that are responsible for sensitization of dogs with atopic dermatitis.
- The high frequency of positive *in vitro* reactions to two or more house dust and/or storage mites in mite-sensitive dogs with atopic dermatitis raises the suspicion of *in vitro* cross-reactivity.
- *In vitro* allergen cross-reactivity should be confirmed by laboratory methods, such as enzyme-linked immunosorbent assay cross-inhibition.

What this paper adds to the field of veterinary dermatology

- Extensive co-sensitization to house dust and storage mites among mite-sensitive dogs with natural atopic dermatitis was demonstrated using intradermal and *in vitro* testing.
- Positive *in vitro* test results to *Dermatophagoides pteronyssinus*, *Acarus siro*, *Tyrophagus putrescentiae* and *Lepidoglyphus destructor* antigens were common among high-IgE-responder beagles that had been experimentally sensitized to *D. farinae*.
- Enzyme-linked immunosorbent assay cross-inhibition demonstrated extensive *in vitro* cross-reactivity between house dust mites, between *D. farinae* and the storage mites *A. siro* and *T. putrescentiae*, and between the latter two mites.

Abstract

***In vitro* cross-reactivity among two house dust (*Dermatophagoides farinae*, *D. pteronyssinus*) and three storage (*Acarus siro*, *Tyrophagus putrescentiae*,**

***Lepidoglyphus destructor*) mites was examined in 20 mite-sensitive dogs with natural occurring atopic dermatitis (group A), 13 high-IgE beagles experimentally sensitized to *D. farinae* (group B), and five healthy beagles (group C). Intradermal testing (IDT) and serology for allergen-specific IgE demonstrated that co-sensitization for all possible pairs of the five mites was generally 45% or higher among group A dogs. In the same dogs, enzyme-linked immunosorbent assay cross-inhibition results indicated that each one of *D. farinae*, *A. siro* and *T. putrescentiae* was a strong inhibitor of all the remaining mites, whereas *D. pteronyssinus* was a strong inhibitor of *L. destructor*. A high number of positive IDT and serology test results for *D. pteronyssinus*, *A. siro*, *T. putrescentiae* and *L. destructor* were recorded among group B dogs. No conclusive evidence of exposure to these mites was found upon analysis of dust samples from their environment and their food for the presence of mites and guanine. Also, the number of positive test results was generally higher among group B than among group C dogs. Enzyme-linked immunosorbent assay cross-inhibition revealed that *D. farinae* was a strong inhibitor of *D. pteronyssinus*, *A. siro* and *T. putrescentiae*. Collectively, these results demonstrated extensive *in vitro* cross-reactivity among house dust and/or storage mites that can explain false-positive results upon testing of dust mite-sensitive dogs with atopic dermatitis.**

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Introduction

House dust mites, such as *Dermatophagoides farinae* (DF) and *D. pteronyssinus* (DP), are important sources of environmental allergens responsible for sensitization of dogs with atopic dermatitis. Their allergenic significance has been established in dogs mainly from a high frequency of positive intradermal testing (IDT) and serology for allergen-specific IgE (serology),¹ but also from mite presence on atopic skin;² the characterization of two important allergens of DF;^{3,4} the ability of sera from atopic dogs to passively transfer DF sensitization to the skin of healthy dogs;⁵ DF-specific proliferation of peripheral blood mononuclear cells from atopic animals;⁶ a positive outcome of provocation testing;⁷ clinical improvement following avoidance;⁸ and a positive response to allergen-specific immunotherapy.⁹

Positive testing (IDT, serology) of dogs with atopic dermatitis to storage mites, such as *Acarus siro* (AS), *Tyrophagus putrescentiae* (TP) and *Lepidoglyphus destructor* (LD), is also frequently encountered.¹ However, the role of these

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mites as sources of the sensitizing environmental allergens remains obscure; storage mites are, for example, rarely detected in the cutaneous microenvironment of both healthy^{10,11} and atopic dogs.¹²

In vitro allergen cross-reactivity is defined as a positive test due to antibodies raised against a similar allergen.¹³ It is usually suspected when simultaneous sensitization to two or more allergens occurs at a frequency higher than that expected by chance alone.¹³ However, since this covariation of sensitization may be the result of parallel sensitization to multiple allergens (different IgE molecules binding to different allergens or epitopes),^{13,14} cross-reactivity should be confirmed by laboratory methods, such as enzyme-linked immunosorbent assay (ELISA) cross-inhibition.^{13,15–17}

Simultaneous sensitization to two or more house dust and/or storage mites may occur in mite-sensitive dogs with atopic dermatitis,¹⁸ depending on the mite species involved. Cross-reactivity has been hypothesized to be higher between DF and DP than between house dust and storage mites or among the various storage mite species.^{18,19} It has also been invoked as a possible explanation for the frequent occurrence of positive test results against mite species not regularly present in the cutaneous microenvironment of atopic dogs.^{2,20} Although some cross-reactivity between different house dust and storage mites has been shown,^{13,21–23} there is scant evidence¹⁵ of its prevalence in dogs with atopic dermatitis. Therefore, the aim of the present study was to examine the extent of cross-reactivity among two house dust (DF, DP) and three storage (AS, TP, LD) mites.

Materials and methods

Dogs

Three groups of dogs were evaluated.

Group A ($n = 20$) was comprised of six male (one castrated) and 14 female (three neutered), mite-sensitive dogs with natural atopic dermatitis. These dogs belonged to 10 different pure breeds (13 of 20) or were cross-breeds with an age range 1–10 years (median = 4.5 years). Canine atopic dermatitis was diagnosed on the basis of compatible history and physical examination; positive IDT and serology; fulfilment of at least three of the major and three of the minor diagnostic criteria proposed by Willemse;²⁴ exclusion of other pruritic skin diseases (e.g. sarcoptic mange, otodectic mange, flea allergic dermatitis, adverse food reactions); or the persistence of clinical pruritus after their elimination (e.g. staphylococcal pyoderma, *Malassezia* dermatitis).^{25,26} Skin scrapings, ear canal parasitological examination, and trial therapy were used to rule out ectoparasites. The possibility of adverse food reaction was eliminated through a 6-week trial with novel-protein home-cooked (8 of 20) or commercial hydrolysed (12 of 20) diet, and concurrent staphylococcal (11 of 20) and/or *Malassezia* (5 of 20) dermatitis were managed with oral antibiotics and ketoconazole, respectively. Additional inclusion criteria were the presence of perennial pruritus, a primarily indoor lifestyle, positive IDT and serology to at least one mite, and no historical evidence of previous parasitic mite infestations (e.g. sarcoptic mange, otodectic mange, cheyletiellosis) that might cause false-positive test reactions.^{27–29} These dogs were originally enrolled for another study investigating the presence of house dust and storage mites in the microenvironment of healthy dogs and mite-sensitive dogs with atopic dermatitis.³⁰

Group B ($n = 13$) comprised six adult (4.75–5.5 years) and seven 5-month-old puppies, belonging to a high-IgE-responder beagle colony⁷ that had been epicutaneously sensitized to DF. There were three intact males and 10 intact females. Experimental sensitization had been achieved by repeated epicutaneous exposure to DF, using an allergen preparation (RMB83M, Greer Laboratories Inc., Lenoir, North Carolina,

USA) of whole bodied, naturally milled DF. This preparation was more than 99% pure, according to the manufacturer, and its allergenic analysis in an external laboratory (Indoor Biotechnologies, Charlottesville, VA, USA) confirmed that it contained trace amounts of Der p 1 ($2.32 \mu\text{g mL}^{-1}$) compared to Der f 1 ($1862 \mu\text{g mL}^{-1}$).⁷ Successful sensitization to DF had been proven, 37–48 days before the experiment, by the emergence of clinical signs typical of canine atopic dermatitis accompanied by an increase of Canine Atopic Dermatitis Extend and Severity Index (median increase = 35.1) after environmental exposure to 50 mg DF allergen preparation,⁷ and by positive serology (ALLERCEPT®, Heska Corporation, Fort Collins, CO, USA) for DF. In addition, these dogs had no history of parasitic mite infestations or experimental exposure to other mites, with the exception of four intradermal injections of the six adult beagles with a 50/50 mixture of DF and DP (Greer Laboratories Inc.) during the preceding 2.5–3.5 years.

Group C ($n = 5$) was comprised of clinically healthy 4- to 5.5-month-old laboratory beagle puppies with no history of skin disease or experimental exposure to house dust or storage mites; they included four intact males and one intact female. These dogs did not belong to high-IgE-responder colony. Group B and C dogs were housed in individual cage runs devoid of any material that could trap dust and previously checked for the absence of mite allergens (MITE-T-Fast™ Allergen Detection System, Aveho Biosciences, Monterey, Tennessee, USA).⁷ Runs were cleaned daily by high-temperature and high-pressure washing. To examine further the possibility of natural exposure of group B and C dogs to mites, as much as possible dust was collected, using a 1440 W vacuum cleaner, from kennel (walls, ceiling, floor and air filters) and dry food containers; also a sample of dry food was manually collected from the bottom of the opened dry food bags currently in use. Dust and food samples were examined for the presence of mites, after mechanical sieving followed by sodium chloride flotation,³¹ and for the guanidine content (Acarex® test, Lifestyle Vertiebsgesellschaft mbH, Wahlstedt, Germany). Three additional household dust samples, randomly selected, were simultaneously analysed (one of them was contaminated with dry dog food particles to macroscopically resemble dry dog food samples), and analyses were performed blindly as to the identity of each sample. Dust samples from the group B and C kennels, their food containers and dry food samples were negative for mites; while $2.6\text{--}119.6 \text{ mites g}^{-1}$ of dust were found in the three household dust samples. Acarex® test was negative for all samples examined.

All procedures were approved by the Institutional Animal Care and Use Committee of the Aristotle University of Thessaloniki, Greece (Group A), and the University of Florida, USA (groups B and C).

Intradermal testing

Intradermal testing was performed according to established procedure.³² Briefly, the lateral thorax was clipped and injection sites were delineated. A volume of 0.05 mL of each allergen or control solution was injected intradermally, and the effects were evaluated after 15–20 min. Topical and systemic medication that may interfere with IDT results had been discontinued for at least 37 days before the procedure, and none of the group A or B dog was undergoing allergen-specific immunotherapy.

Group A dogs were sedated with $30 \mu\text{g kg}^{-1}$ medetomidine (Dormitor®, Pfizer-Orion Corporation, Espoo, Finland) given intramuscularly. Mite allergen extracts and the relevant positive and negative controls from two manufacturers were used: DF and DP from Greer Laboratories Inc. and AS, TP and LD from Artu Biologicals N.V. (Lelystad, the Netherlands). AS, TP and LD allergen extracts were injected at the recommended dilution ($100 \text{ Noon units mL}^{-1}$), whereas DF and DP were used at three different dilutions ($1/5000$, $1/25\,000$ and $1/100\,000 \text{ w v}^{-1}$) in phosphate-buffered saline containing 0.4% phenol. The cutaneous reactions were evaluated by a single investigator (RF) with knowledge of the identity of each extract, and scored from 0 to +4 using the objective method.²⁴ Wheals with a mean diameter equal or greater than the halfway between the mean diameters of the positive control and the negative control were scored $\geq +2$ and were considered positive.

Group B and C dogs were not sedated for IDT. All five mite extracts used in group B and C dogs were from the same source (Greer Laboratories Inc.), and were tested at three different dilutions ($1/5000$,

1/25 000, and 1/100 000 w v⁻¹). Size, erythema, and turgidity of the wheals were first evaluated subjectively and independently by two investigators (MNS, RM) in comparison to those of the positive and negative controls, with no knowledge of the identity of the allergen extracts (only of the injection sites of the positive and negative controls) and subsequently evaluated by MNS with the objective method. Subjective and objective scores were recorded separately. Again, reactions $\geq +2$ were considered positive.

Serology for allergen-specific IgE

Serum samples were collected for determination of mite allergen-specific IgE by ELISA. For groups A and B, two different methods of serology were employed. In the first method the biotinylated recombinant extracellular alpha chain of the human high-affinity IgE receptor (FcεR1α) was used to detect allergen-specific IgE against DF, DP and TP (ALLERCEPT®, Heska Laboratories, Switzerland for group A; Heska Corporation, Fort Collins, CO, USA for group B). With the second method (Greer Laboratories Inc.) a mixture of biotinylated monoclonal anticanine IgE antibodies was used for the detection of allergen-specific IgE against all five mites. For group C dogs, only the latter method was employed. Results of serology were classified as positive or negative according to the interpretation of the relevant commercial laboratory.

Enzyme-linked immunosorbent assay cross-inhibition

Serum samples from 15 dogs in group A and eight group B dogs were selected, based on their relatively high IgE reactivity against more than one mite by the second serology method.

For ELISA cross-inhibition, the IgE reactivity against each mite (DF, DP, AS, TP and LD) was determined and the mite protein required to effect a 50% reduction in specific reactivity was quantified in each instance using a modification of previously described inhibition protocol.³³ Briefly, a seven-tube serial threefold dilution of each mite extract was prepared and 50 µL of each dilution was transferred to duplicate wells that had been previously coated with saturating levels of mite allergen; reciprocal inhibitions were completed by evaluating these liquid phase allergens against each of the mite extracts that were individually coated to wells. Then 50 µL of positive control sera were added to each well, except those allotted to negative control and background. Following overnight incubation at 4–8 °C, antibody binding was detected using a monoclonal cocktail of biotinylated anti-IgE second antibodies, streptavidin–alkaline phosphatase enzyme conjugate, and p-nitrophenylphosphate substrate. Substrate development was stopped using 20 mM cysteine and the absorbance of each well was measured at 405 nm using an automated plate reader. The relative amount of liquid phase allergen required to effect 50% inhibition in response was interpolated from a regression curve constructed from the varying dose–responses and presented as a comparative ratio of homologous solid phase antigen. The dose–response curve was determined using only the dilutions of extract that yields inhibition between 10 and 90%, and 50% inhibition was spanned within this range. If 50% inhibition was not evident, results were expressed as greater than the maximum concentration of allergen used (3750 ng mL⁻¹) for the inhibition evaluation; if greater than 50% inhibition was evident with the minimum concentration of allergen use, the results were expressed as less than the minimum concentration (10 ng mL⁻¹). Mite allergens used in the assays were derived from 99% (v/v) pure mite bodies, verified as to their identity and purity before extraction.

Based on the ratio of inhibiting (liquid phase) to homologous (solid phase) allergen required for 50% inhibition, cross-reactivity was considered little to absent if the ratio was > 10 , low to moderate if the ratio was 3–10 and substantial if the ratio was ≤ 3 . Any given mite was considered a strong inhibitor of another mite when substantial cross-reactivity was found in 50% or more of serum samples, provided that at least four samples were tested.

Statistical analysis

For group A dogs, co-sensitization to each pair of mites was calculated by dividing the number of dogs with a positive test result (IDT or serology) to both mites by the number with a positive result to either mite.

The frequency of positive IDT and serology results to DP was compared between the puppies and adults in Group B with Fisher's exact test. The same test was also used to compare the frequency of positive allergy testing results between group B and C dogs.

After combining IDT results of group B and group C dogs, the frequency of IDT reactions to each mite dilution was compared between the objective and either of the two subjective evaluations with McNemar's test.³⁴ The agreement among these three methods of IDT interpretation was examined by calculating Cohen's kappa coefficient (κ). Agreement was considered poor if $\kappa \leq 0.20$, fair if $0.21 \leq \kappa \leq 0.40$, moderate if $0.41 \leq \kappa \leq 0.60$, substantial if $0.61 \leq \kappa \leq 0.80$, and good if $\kappa \geq 0.80$.³⁴

The frequency of substantial inhibition (ratio of inhibiting to homologous allergen required for 50% inhibition ≤ 3) for each pair of mite allergens was compared between groups A and B using χ^2 test.

All statistical analyses were performed in SPSS 13.3 (SPSS Inc., Chicago, IL, USA) for Windows, and *P*-values less than 0.05 were considered significant.

Results

The frequency of positive IDT and serology results of group A dogs to the mite allergens is shown on Table 1. Based on IDT, co-sensitization at a frequency $\geq 50\%$ was observed between DF and DP, between DF and the three storage mites (AS, TP and LD), between DP and the three storage mites, as well as among the storage mites. Based on serology, the frequency of co-sensitization ranged from 45 to 100% for all possible pairs of mites (Table 2). ELISA cross-inhibition revealed that: (i) DF was strong inhibitor of IgE reactivity against the remaining four mites (DP, AS, TP and LD); (ii) DP was strong inhibitor of LD; (iii) AS was strong inhibitor of the remaining four mites (DF, DP, TP and LD); (iv) TP was strong inhibitor of the remaining four mites (DF, DP, AS and LD); and (v) LD was not strong inhibitor of any mite tested (Table 3).

The frequency of positive IDT and serology results of group B dogs to the mite allergens is shown in Table 4. Irrespective of investigator and the method of IDT interpretation, $\geq 50\%$ of the dogs reacted to the higher concentration (1/5000 w v⁻¹) of most mite allergens. In addition, serology to all mite allergens, except LD, was positive in $> 75\%$ of the cases (Table 4). The frequency of positive testing results to DP did not differ between puppies and adult high-IgE-responder beagles. ELISA cross-inhibition revealed that: (i) DF was a strong inhibitor of IgE reactivity against three mites (DP, AS and TP); (ii) AS was strong inhibitor of LD; and (iii) TP was strong inhibitor of AS (Table 3).

Group C dogs did not react to any mite extract when IDT was evaluated with the objective method. Only one of them presented a few weak (+2) positive reactions (against AS at 1/25 000 w v⁻¹, AS at 1/100 000 w v⁻¹ and LD at 1/5000 w v⁻¹ according to the first investigator and against DP at 1/25 000 w v⁻¹ and LD at 1/100 000 w v⁻¹ according to the second investigator) when IDT was evaluated subjectively. With serology using a mixture of monoclonal anticanine IgE antibodies, one dog (20%) reacted to AS and two dogs (40%) to TP.

The frequency of positive testing results was significantly lower in group C compared to group B dogs for most mite allergens (Table 4). Also, when group B and C dogs were considered together, there was no significant difference in the frequency of positive IDT results between the objective and either of the two subjective evaluations, and the

Allergen	Frequency (%) of positive results
Intradermal testing	
<i>Dermatophagoides farinae</i>	
1/5000 w v ⁻¹	20/20 (100%)
1/25 000 w v ⁻¹	15/20 (75%)
1/100 000 w v ⁻¹	5/20 (25%)
<i>Dermatophagoides pteronyssinus</i>	
1/5000 w v ⁻¹	10/20 (50%)
1/25 000 w v ⁻¹	2/20 (10%)
1/100 000 w v ⁻¹	0/20
<i>Acarus siro</i>	13/20 (65%)
<i>Tyrophagus putrescentiae</i>	14/20 (70%)
<i>Lepidoglyphus destructor</i>	11/20 (55%)
Serology for allergen-specific IgE	
	Laboratory no. 1
	Laboratory no. 2
<i>Dermatophagoides farinae</i>	20/20 (100%)
<i>Dermatophagoides pteronyssinus</i>	14/20 (70%)
<i>Acarus siro</i>	NT
<i>Tyrophagus putrescentiae</i>	20/20 (100%)
<i>Lepidoglyphus destructor</i>	NT

Laboratory no. 1: detection of allergen-specific IgE using biotinylated recombinant extracellular alpha chain of human high-affinity IgE receptor; Laboratory no. 2: detection of allergen-specific IgE using a mixture of monoclonal anticanine IgE antibodies; NT: not tested.

Table 2. Frequency (%) of co-sensitization among five house dust and storage mites based on the results of intradermal and serology testing for allergen-specific IgE in 20 dust-mite sensitive dogs with natural atopic dermatitis (group A)

Intradermal testing						
Allergen	DP*	DP†	DP‡	AS§	TP§	LD§
DF*	10/20 (50%)	–	–	13/20 (65%)	14/20 (70%)	11/20 (55%)
DF†	–	2/14 (14.3%)	–	10/16 (62.5%)	11/16 (68.8%)	9/15 (60%)
DF‡	–	–	0/5	5/12 (41.7%)	5/13 (38.5%)	4/11 (36.4%)
DP*	–	–	–	9/14 (64.3%)	9/15 (60%)	8/13 (61.5%)
DP†	–	–	–	2/13 (15.4%)	2/14 (14.3%)	2/11 (18.2%)
DP‡	–	–	–	0/13	0/14	0/11
AS§	–	–	–	–	13/14 (92.9%)	11/13 (84.6%)
TP§	–	–	–	–	–	11/14 (78.6%)
Serology for allergen-specific IgE						
Allergen	DP	AS			TP	LD
Laboratory no. 1						
DF	14/20 (70%)	NT			20/20 (100%)	NT
DP	–	NT			14/20 (70%)	NT
AS	–	–			NT	NT
TP	–	–	–			NT
Laboratory no. 2						
DF	9/19 (47.4%)	19/20 (95%)			19/20 (95%)	10/19 (52.6%)
DP	–	9/20 (45%)			9/20 (45%)	6/13 (46.2%)
AS	–	–			20/20 (100%)	10/20 (50%)
TP	–	–			–	10/20 (50%)

*1/5000 w v⁻¹; †1/25 000 w v⁻¹; ‡1/100 000 w v⁻¹; §100 Noon units mL⁻¹.

AS, *Acarus siro*; DF, *Dermatophagoides farinae*; DP, *Dermatophagoides pteronyssinus*; Laboratory no. 1: detection of allergen-specific IgE using biotinylated recombinant extracellular alpha chain of human high-affinity IgE receptor; Laboratory no. 2: detection of allergen-specific IgE using a mixture of monoclonal anticanine IgE antibodies; LD, *Lepidoglyphus destructor*; NT, not tested; TP, *Tyrophagus putrescentiae*.

agreement among these methods was moderate ($\kappa = 0.465$ between the objective method and first investigator; $\kappa = 0.516$ between the objective method and second investigator; $\kappa = 0.482$ between the two investigators).

The frequency of substantial inhibition of IgE reactivity, upon ELISA cross-inhibition, was significantly higher for group A compared to group B, regarding the: (i) inhibition of DF by AS and TP; (ii) inhibition of DP by AS and TP; and (iii) inhibition of TP by DF.

Discussion

The inclusion criteria of group A dogs were selected to ensure, not only the diagnosis of atopic dermatitis, but also their natural sensitization to house dust and/or storage mites (indoor lifestyle, perennial pruritus, no historical evidence of parasitic mite infestations). By the time these dogs were enrolled, AS, TP, and LD allergen extracts for IDT were not commercially available from Greer Laboratories

Table 1. Frequency of positive results using intradermal and serology testing for allergen-specific IgE in 20 dust mite-sensitive dogs with natural atopic dermatitis (group A)

Table 3. Cross-reactivity among five house dust and storage mites evaluated by ELISA cross-inhibition in serum samples from 15 mite-sensitive dogs with natural atopic dermatitis (group A) and eight high-IgE-responder beagles that had been experimentally sensitized to *Dermatophagoides farinae* (group B)

Allergen		Ratio of inhibiting to homologous allergen for 50% inhibition*					
		Natural atopic dermatitis			High-IgE-responder beagles		
		≤ 3	3–10	> 10	≤ 3	3–10	> 10
DF	DP	1/12 (8.3%)	0/12	11/12 (91.7%)	2/7 (28.6%)	2/7 (28.6%)	3/7 (42.9%)
	AS†	8/12 (66.7%)	2/12 (16.7%)	2/12 (16.7%)	0/7	0/7	7/7 (100%)
	TP†	7/12 (58.3%)	4/12 (33.3%)	1/12 (8.3%)	0/7	1/7 (14.3%)	6/7 (85.7%)
	LD	0/12	0/12	12/12 (100%)	0/7	0/7	7/7 (100%)
DP	DF	5/5 (100%)	0/5	0/5	6/6 (100%)	0/6	0/6
	AS†	5/5 (100%)	0/5	0/5	1/4 (25%)	0/4	3/4 (75%)
	TP†	5/5 (100%)	0/5	0/5	1/4 (25%)	0/4	3/4 (75%)
	LD	1/5 (20%)	1/5 (20%)	3/5 (60%)	0/4	0/4	4/4 (100%)
AS	DF	11/15 (73.3%)	2/15 (13.3%)	2/15 (13.3%)	7/8 (87.5%)	0/8	1/8 (12.5%)
	DP	3/13 (23.1%)	3/13 (23.1%)	7/13 (53.8%)	3/8 (37.5%)	2/8 (25%)	3/8 (37.5%)
	TP	15/15 (100%)	0/15	0/15	7/8 (87.5%)	0/8	1/8 (12.5%)
	LD	3/13 (23.1%)	0/13	10/13 (76.9%)	1/6 (16.7%)	0/6	5/6 (83.3%)
TP	DF†	11/15 (73.3%)	1/15 (6.7%)	3/15 (20%)	4/7 (57.1%)	1/7 (14.3%)	2/7 (28.6%)
	DP	0/14	2/14 (14.3%)	12/14 (85.7%)	1/7 (14.3%)	2/7 (28.6%)	4/7 (57.1%)
	AS	10/15 (66.7%)	3/15 (20%)	2/15 (13.3%)	1/6 (16.7%)	3/6 (50%)	2/6 (33.3%)
	LD	2/14 (14.3%)	1/14 (7.1%)	11/14 (78.6%)	0/6	0/6	6/6 (100%)
LD	DF	4/5 (80%)	0/5	1/5 (20%)	1/1 (100%)	0/1	0/1
	DP	3/5 (60%)	1/5 (20%)	1/5 (20%)	1/1 (100%)	0/1	0/1
	AS	5/5 (100%)	0/5	0/5	5/5 (100%)	0/5	0/5
	TP	4/5 (80%)	0/5	1/5 (20%)	1/1 (100%)	0/1	0/1

*Cross-reactivity was considered little to absent if ratio was > 10, low to moderate if ratio was 3–10 and substantial if ratio was ≤ 3.

†Significantly ($P < 0.05$) higher frequency of substantial ELISA cross-inhibition (ratio of inhibiting to homologous allergen for 50% inhibition ≤ 3) for dust mite-sensitive dogs with natural atopic dermatitis (group A) than for high-IgE-responder beagles that had been experimentally sensitized to *Dermatophagoides farinae* (group B).

AS, *Acarus siro*; DF, *Dermatophagoides farinae*; DP, *Dermatophagoides pteronyssinus*; LD, *Lepidoglyphus destructor*; TP, *Tyrophagus putrescentiae*.

Inc.; for this reason storage mite allergen extracts from a second manufacturer (Artu Biologicals N.V.) were used. Since these extracts were supplied as ready-to-use solutions, it was not possible to test them in multiple dilutions. Also, their allergenic content was expressed differently (Noon units) than the allergenic content of house dust mite allergen extracts (w v^{-1}), thus prohibiting any direct comparison of IDT results between house dust and storage mites. House dust mite allergen extracts were tested in multiple dilutions because there is no consensus on their optimal concentration for IDT³⁵ with suggested concentrations ranging from 1/5000 to 1/75 000 w v^{-1} .^{36,37} Nevertheless, the higher frequency of positive IDT and serology

results to DF than DP, as well as the high frequency of positive test results to storage mites, was expected, based on previous studies.^{1,5,8,15,18,22,38–43}

The results clearly show that dogs with atopic dermatitis are frequently co-sensitized to house dust and storage mites. This could be the result of parallel exposure and sensitization to different mite species and/or of *in vitro* cross-reactivity. The frequency of co-sensitization between DF and DP, as well as between either DF or DP and the three storage mites, generally declined as the concentration of house dust mite allergen extracts used in IDT was decreased (Table 2). This was the result of the inconsistency of IDT reactions to the higher dilutions of house dust

Table 4. Frequency of intradermal testing and serology for allergen-specific IgE-positive results of 13 high-IgE-responder beagles that had been experimentally sensitized to *Dermatophagoides farinae* (group B)

Allergen	Frequency (%) of positive results		
	Intradermal testing		
	Subjective method		
	First investigator	Second investigator	Objective method
DF†	11/13 (84.6%)*	12/13 (92.3%)*	12/13 (92.3%)*
DF‡	7/13 (53.8%)	8/13 (61.5%)*	10/13 (76.9%)*
DF§	5/13 (38.5%)	3/13 (23.1%)	8/13 (61.5%)*
DP†	13/13 (100%)*	13/13 (100%)*	11/13 (84.6%)*
DP‡	7/13 (53.8%)	10/13 (76.9%)*	10/13 (76.9%)*
DP§	5/13 (38.5%)	6/13 (46.2%)	8/13 (61.5%)*
AS†	7/13 (53.8%)	4/13 (30.8%)	9/13 (69.2%)*
AS‡	5/13 (38.5%)	6/13 (46.2%)	6/13 (46.2%)
AS§	0/13	3/13 (23.1%)	3/13 (23.1%)
TP†	9/13 (69.2%)*	10/13 (76.9%)*	8/13 (61.5%)*
TP‡	6/13 (46.2%)	7/13 (53.8%)	6/13 (46.2%)
TP§	4/13 (30.8%)	7/13 (53.8%)	7/13 (53.8%)
LD†	8/13 (61.5%)	6/13 (46.2%)	8/13 (61.5%)*
LD‡	4/13 (30.8%)	7/13 (53.8%)	8/13 (61.5%)*
LD§	1/13 (7.7%)	5/13 (38.5%)	7/13 (53.8%)
Serology for allergen-specific IgE			
	Laboratory no. 1	Laboratory no. 2	
DF	13/13 (100%)	12/13 (92.3%)*	
DP	13/13 (100%)	10/13 (76.9%)*	
AS	NT	11/13 (84.6%)*	
TP	10/13 (76.9%)	11/13 (84.6%)	
LD	NT	6/13 (46.2%)	

†1/5000 w v⁻¹; ‡1/25 000 w v⁻¹; §1/100 000 w v⁻¹.*Significantly ($P < 0.05$) higher frequency of positive intradermal test or serology for allergen-specific IgE (laboratory no. 2) results compared to clinically healthy dogs (group C). Group C dogs were not tested by laboratory no. 1; thus statistical analysis was not performed.AS, *Acarus siro*; DF, *Dermatophagoides farinae*; DP, *Dermatophagoides pteronyssinus*; Laboratory no. 1: detection of allergen-specific IgE using biotinylated recombinant extracellular alpha chain of human high-affinity IgE receptor; Laboratory no.2: detection of allergen-specific IgE using a mixture of monoclonal anticanine IgE antibodies; LD, *Lepidoglyphus destructor*; NT: not tested; TP, *Tyrophagus putrescentiae*.

mite extracts, such as DF at 1/100 000 w v⁻¹, and DP at 1/25 000 w v⁻¹ and 1/100 000 w v⁻¹ (Table 1). If these three dilutions are not taken into account, then IDT-based co-sensitization for all possible pairs of the five mites would be 50% or higher. In a similar way, serology-based co-sensitization was always 45% or higher. These figures correlate well with the results of previous studies showing that co-sensitization to DF and DP ranges from 21.9 to 79.6%, depending on the method of IDT interpretation,⁴⁴ that the odds ratio of co-sensitization to DF and DP is high (12.9) based on IDT results,⁴² and that there is significant correlation between IDT or serology results for these two mites,⁴¹ and they are also similar with findings in humans with atopic dermatitis.⁴⁵ In addition, although exact figures cannot be calculated from previous studies, frequent co-sensitization to house dust and storage mites,^{18,41,43,46} as well as to AS and TP¹⁸, is evident in dogs with atopic dermatitis, like in humans with atopic dermatitis.^{13,45,47}

In humans with atopic dermatitis the high homology between DF and DP, evident by the presence of up to 21 cross-reacting allergens,²¹ results in extended cross-reactivity,^{13,48} and the same has been suggested for atopic dogs based on the presence of 16 cross-reacting allergens²² and the results of ELISA cross-inhibition,¹⁵ although the cross-reactivity may be less than in humans.²² In the present study, DF was a strong inhibitor of IgE reactivity against DP, whereas the reverse was not true,

indicating that false-positive results are more likely to occur against DP in DF-sensitive dogs than against DF in DP-sensitive dogs with atopic dermatitis.

High molecular weight allergen (Der f 15), a heavily glycosylated chitinase, is the most important allergen of DF.^{3,19,49} Anecdotally, a similar but less glycosylated chitinase is also a major DP allergen.^{19,20} If anti-Der f 15/anti-Der p 15 IgE antibodies are directed against glycoprotein residues, there are two, nonmutually exclusive explanations for the comparatively higher inhibitory potential of DF against DP: (i) a substantial amount of DF-specific IgE antibodies are directed against glycoproteins that are not present in DP, and (ii) the inhibitory potential of DP against DF has been underestimated because the allergen quantities used in ELISA cross-inhibition have been expressed in terms of their protein content and, thus, do not take into account their carbohydrates. The latter is an inherent drawback of this methodology when nonprotein substances play a major role in inhibition.⁵⁰ Also, generalization of these findings should be viewed with scepticism because results of inhibition studies depend on the allergen extracts used,⁵¹ and on the mite species responsible for the sensitization of the atopic patients.¹³ Group A dogs had been mainly exposed to DF than to DP,³⁰ consequently reciprocal inhibition results could have been different if serum samples of mite-sensitive dogs from areas where DP is the predominant house dust mite had been examined.

Nevertheless, these results show an extensive cross-reactivity between DF and DP in mite-sensitive dogs with natural atopic dermatitis.

Cross-reactivity between house dust and storage mites has been proven in humans with atopic dermatitis^{21,52–56} and some cross-reacting allergens have been identified.^{13,21,55,57} Considering cross-reactivity between house dust and individual storage mites the results of human allergy studies are contradictory; some of them show higher cross-reactivity between house dust mites and LD than between house dust mites and AS or TP,⁴⁵ whereas other studies show exactly the opposite.¹³ A similar situation has been suggested for dogs with atopic dermatitis⁵⁸ and confirmed by the results of the present study. Our findings imply that false-positive test results may be mainly expected for AS, TP, and LD in atopic dogs sensitive to DF, for LD in dogs sensitive to DP and for both house dust mites in dogs sensitive to AS and/or TP.

In both humans and dogs with atopic dermatitis, cross-reactivity among storage mites has been considered relatively limited^{13,18,40,53} and has been mainly attributed to similarities of group II allergens among different storage mite species. In group A dogs, in contrast to atopic humans,⁵³ AS and TP were strong inhibitors of each other as well as of LD, whereas LD was not a strong inhibitor of AS or TP. The higher reciprocal cross-reactivity between AS and TP may be explained by their closer taxonomic relationship and antigen homology compared to LD;^{23,59} alternatively it could also be explained by sensitization of these dogs to AS and/or TP but not to LD, leading to the production of a repertoire of IgE antibodies, with some of them being cross-reactive with LD and others being AS-specific and/or TP-specific. Nevertheless, these findings may explain false-positive allergy testing results for TP and LD in atopic dogs sensitive to AS and for AS and LD in dogs sensitive to TP.

Experimentally sensitized high-IgE-responder beagles (group B) have been proven to be a useful model of canine atopic dermatitis, closely mimicking natural disease clinically, histopathologically and immunologically.^{7,60} Blinding of the investigators on the identity of allergen extracts was of particular importance, in order to avoid the inherent observer bias during IDT evaluation in dogs known to be sensitive to a specific mite (DF). In previous studies a higher number of IDT reactions was recorded with the subjective than the objective method of interpretation,^{61,62} which is not in agreement with our findings. The discrepancy could be partially explained by differences in the definition of objective scoring positive reactions: in previous studies to consider a reaction as positive it was necessary to present erythema⁶² or the mean wheal diameter to be higher (not equal or higher) than the halfway of the mean diameter of the negative and positive controls.⁶¹ Furthermore, the high interobserver variation that is inherent to the subjective method of interpretation,³⁸ even when just one criterion, such as erythema, is evaluated,⁶³ can easily explain such discrepancies, as well as the moderate agreement of the three IDT interpretations in the present study.

The high frequency of positive IDT results to mite extracts among group B dogs cannot be attributed to irritant reactions, because the frequency of IDT reactions was generally significantly higher compared to group C for

the more concentrated (1/5 000 w v⁻¹) mite extracts, and thus more likely to produce irritant reactions. Similarly, the frequency of positive serology was significantly higher among group B than group C dogs for three mites (DF, DP and AS). Therefore, it can be concluded that the positive test results in group B dogs were due to skin-bound and circulating IgE molecules recognizing mite allergens.

Positive test results in group B dogs to DF were obviously the result of their experimental sensitization to this mite. Considering the remaining four mites, possible explanations, besides mite cross-reactivity, may include: (i) previous IDT of adult group B dogs with a mixture of DF and DP that could possibly have induced reactions to DP;⁶⁴ However, this cannot explain the positive allergy testing results of group B puppies against DP at a frequency that was not different from that in the adult dogs; (ii) contamination by DP of the DF preparation that had been used for the experimental sensitization. However, the detection of trace amount of Der p 1 in this preparation may also be explained by its cross-reactivity with Der f 1, due to the 80–85% identity in their chemical structure;^{13,15} (iii) natural exposure to DP and storage mites that could have been present in the environment and/or the food of group B dogs at a level lower than the sensitivity limits of the three methods employed for the detection of mites, their allergens and guanine. Although the latter cannot be dismissed, cross-reactivity between DF and the remaining mite species seems the most logical explanation for the high number of positive IDT and serology results against DP, AS, TP and LD. This is further supported by the results of ELISA cross-inhibition showing that DF was a strong inhibitor of DP, AS and TP, whereas, contrary to group A dogs, the reverse was not true.

Conclusions

The results demonstrate that mite-sensitive dogs with natural atopic dermatitis show co-sensitization to house dust and storage mites. In addition, high-IgE-responder beagles that had been experimentally sensitized to *D. farinae*, frequently gave positive intradermal and serology test results to *D. pteronyssinus*, *A. siro*, *T. putrescentiae* and *L. destructor*. ELISA-cross inhibition proved extensive *in vitro* cross-reactivity between house dust mites, between *D. farinae* and the storage mites *A. siro* and *T. putrescentiae*, and between the latter two mites. Further studies are needed to examine if *in vivo* cross-reactivity may also occur among these mites – i.e. the appearance of clinical signs upon exposure to a naïve but cross-reacting allergen.

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Résumé Cette étude s'est intéressée à évaluer la réactivité croisée *In vitro* entre deux acariens des poussières (*Dermatophagoides farinae*, *D. pteronyssinus*) et trois acariens de stockage (*Acarus siro*, *Tyrophagus putrescentiae*, *Lepidoglyphus destructor*) chez 20 chiens atopiques sensibilisés aux acariens (groupe A), 13 Beagles haut répondeurs en IgE sensibilisés expérimentalement à *D. farinae* (groupe B), et 5 Beagles sains (groupe C). Les tests intradermiques (IDT) et sérologiques pour les IgE spécifiques d'allergènes ont démontré qu'une co-sensibilisation pour toutes les paires possibles d'acariens était généralement de 45% ou plus pour les chiens du groupe A. Pour les mêmes chiens, les tests de cross-inhibition ELISA ont indiqué que *D. farinae*, *A. siro* et *T. putrescentiae* étaient des inhibiteurs de tous les autres acariens alors que *D. pteronyssinus* était un inhibiteur de *L. destructor*. Un nombre élevé de résultats positifs en IDT et en sérologie étaient observés dans le groupe B pour *D. pteronyssinus*, *A. siro*, *T. putrescentiae* et *L. destructor*. Aucun argument en faveur d'une exposition à ces acariens n'a été trouvé en analysant des prélèvements de poussières depuis leur environnement ou leur nourriture à la recherche d'acariens ou de guanine. En outre, le nombre de tests positifs était généralement plus élevé parmi les chiens du groupe B par rapport aux chiens du groupe C. Les tests ELISA d'inhibition ont montré que *D. farinae* était un inhibiteur de *D. pteronyssinus*, *A. siro*, et *T. putrescentiae*. Globalement, ces résultats démontrent des réactions croisées importantes entre les acariens des poussières et de stockage qui peuvent expliquer les résultats faux positifs observés en testant les chiens atopiques.

Resumen Se examinó la reactividad cruzada entre dos ácaros del polvo (*Dermatophagoides farinae*, *D. pteronyssinus*) y tres ácaros del almacenamiento (*Acarus siro*, *Tyrophagus putrescentiae*, *Lepidoglyphus destructor*) en 20 perros sensibilizados a ácaros y padeciendo dermatitis atópica natural (grupo A), 13 perros Beagle con IgE elevada y experimentalmente sensibilizados a *D. farinae* (grupo B), y cinco perros Beagle sanos (grupo C). La prueba intradérmica (IDT) y la serología de IgE específica de alérgeno demostró sensibilización cruzada para todas las parejas posibles de los cinco ácaros fue generalmente 45% o mayor entre los perros del grupo A. En estos mismos perros, los resultados del ensayo de inhibición cruzada en inmunoabsorción ligada a enzimas indicaron que cada uno de los ácaros *D. farinae*, *A. siro* y *T. putrescentiae* era un inhibidor fuerte de *L. destructor*. Un elevado número de animales positivos a la prueba intradérmica y a la serología para *D. pteronyssinus*, *A. siro*, *T. putrescentiae* y *L. destructor* se encontraron entre los animales del grupo B. No se encontró evidencia de exposición a estos ácaros tras el análisis del polvo del ambiente y de su

comida para la presencia de ácaros y guanina. Además el número de resultados positivos de las pruebas fue generalmente más elevado en perros del grupo B que en perros del grupo C. La inhibición cruzada en inmunoabsorción ligada a enzimas indicó que *D. farinae* fue un inhibidor fuerte de *D. pteronyssinus*, *A. siro* and *T. putrescentiae*. De forma colectiva, estos resultados demuestran una extensa reacción cruzada in vitro entre los ácaros del polvo y los ácaros del almacenamiento que podría explicar los falsos positivos al analizar perros sensibilizados a ácaros del polvo con dermatitis atópica.

Zusammenfassung Die *in-vitro* Kreuzreaktivität zwischen zwei Hausstaubmilben (*Dermatophagoides farinae*, *D. pteronyssinus*) und drei Futtermittelmilben (*Acarus siro*, *Tyrophagus putrescentiae*, *Lepidoglyphus destructor*) wurde bei 20 auf Milben allergischen Hunden mit natürlich auftretender atopischer Dermatitis (Gruppe A), bei 13 Beagles mit hohem IgE-Gehalten, die experimentell auf *D. farinae* sensibilisiert worden waren (Gruppe B) und bei 5 gesunden Beagles (Gruppe C) untersucht. Intradermaltests (IDT) und Serologie für Allergen-spezifisches IgE zeigten, dass die Co-Sensibilisierung für alle möglichen Paare der fünf Milben in der Gruppe A der Hunde generell bei 45% oder höher war. Bei denselben Hunden wiesen die Ergebnisse der Kreuz-Inhibition des Enzyme-linked Immunosorbent Assay darauf hin, dass sowohl *D. farinae*, als auch *A. siro* und *T. putrescentiae* starke Inhibitoren für alle übrigen Milben waren, während *D. pteronyssinus* ein starker Inhibitor für *L. destructor* war. Eine große Anzahl an positiven IDT und Serologie Testergebnissen für *D. pteronyssinus*, *A. siro*, *T. putrescentiae* und *L. destructor* wurden in der Gruppe B der Hunde gefunden. Bei der Analyse von Staubproben aus ihrer Umgebung und bei der Analyse ihres Futters auf das Vorhandensein von Milben und Guanin wurden im Bezug auf die Exposition zu diesen Milben keine schlüssigen Anhaltspunkte gefunden. Ebenso war die Anzahl der positiven Testergebnisse generell in der Gruppe B höher als bei den Hunden der Gruppe C. Die Kreuz-Inhibition des Enzyme-linked Immunosorbent Assay zeigte, dass *D. farinae* ein starker Inhibitor für *D. pteronyssinus*, *A. siro* und *T. putrescentiae* war. Zusammengefasst zeigten diese Ergebnisse eine ausgedehnte *in-vitro* Kreuzreaktion unter den Hausstaubmilben und/oder den Futtermittelmilben, welche falsch-positive Ergebnisse beim Testen von Hausstaubmilben-sensitiven Hunden mit atopischer Dermatitis erklären können.