

An ELISA for recombinant *Lepidoglyphus destructor*, Lep d 2, and the monitoring of exposure to dust mite allergens in farming households

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Summary

Background Exposure to indoor allergens, such as dust mites, has been recognized as a risk factor for sensitization and symptoms.

Objective To develop a two-site ELISA for the determination of Lep d 2 in the reservoir, to measure dust mite allergen exposure (Lep d 2, Der p 1, Der f 1 and Der 2) in farm households, and to investigate whether exposure to these allergens is associated with sensitization, asthma and rhinoconjunctivitis.

Methods Monoclonal antibodies to recombinant (r)Lep d 2 were produced with standard hybridoma technique. Dust samples from 393 households were analysed for allergen content by two-site ELISA methods.

Results A two-site Lep d 2 ELISA was developed with a detection limit of 0.09 µg/g. The assay was highly reproducible and levels of Lep d 2 showed a strong correlation with the number of *Lepidoglyphus* mites (r_s : 0.7; $P=0.0002$). Lep d 2 was detected in 20% of the homes; levels ranged from 0.09 to 1.7 µg/g of dust. Der p 1 was recorded in 59% of the samples, ranging from 0.055 to 139 µg/g, and Der f 1 and Der 2 in 40% and 50% of the samples, ranging from 0.055 to 24.5 µg/g and 24.3 µg/g, respectively. *Dermatophagoides* allergens were significantly higher in mattresses than in carpets ($P<0.0001$), but this difference was not observed with Lep d 2. A strong relationship between immunoglobulin (Ig)E to rLep d 2 and asthma (OR = 10.4) and rhinoconjunctivitis (OR = 7.5) was seen. Furthermore, sensitization to *D. pteronyssinus* was significantly associated with asthma (OR: 13.7) and rhinoconjunctivitis (OR: 5.7).

Conclusion When assessing mite allergen exposure in rural homes, not only the Der p 1, Der f 1 and Der 2 allergens, but also the Lep d 2 allergen should be taken into consideration.

Keywords ELISA, mite allergen, exposure, sensitization, symptoms

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Introduction

Exposure to mite allergens, both from house dust mites and from storage mites, has been recognized as an important risk factor for the development of sensitization and allergic disease [1–7]. House dust mites are mainly found indoors whereas the storage mites, which feed on moulds, have a wider range of habitat and have been found in all kind of localities where organic material is stored, as well as in dwellings. During recent years there has been an increased awareness of the importance of storage mites as a cause of sensitization in urban dwellings [7–9]. Hence, the term dust mite is now commonly used to describe mites in the indoor environment, and includes both house dust mites and storage mites. Several species of storage mites exist, but *Lepidoglyphus destructor* seems to be one of the most common species in Europe. It dominates in dust samples

from farming environments [5,10]. In earlier studies regarding respiratory diseases among farmers on the Swedish island of Gotland, we have shown that the storage mite *L. destructor* is a major cause of allergic reactions in the farming environment, being more prevalent than the house dust mite *Dermatophagoides pteronyssinus* [4,6]. Most studies concerning mite allergy among farmers have focused on investigating sensitization to storage mites and have examined the mite fauna in the working areas of the farms. Few studies have explored the mite fauna of the indoor environment of farmers' homes. Iversen and colleagues [5], who counted mites in dust from mattresses in 105 farmhouses, found high numbers of both house dust mites and storage mites. Franz and colleagues [11] also recorded both storage and house dust mites in dust collected from the living areas of 20 farms in Germany. However, the levels of mite allergen exposure among farmers are even less investigated. Hitherto, only one study, by Radon and colleagues [12], has measured the concentration of the house dust mite allergens Der p 1, Der f 1 and Der 2, and of Lep d 2, the major allergen of *L. destructor*, in the farming environment. We have recently cloned and expressed Lep d 2 as a recombinant

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protein, rLep d 2, and have shown that it possesses IgE reactivity *in vitro* as well as *in vivo* [13–16]. rLep d 2 can now be produced in large amounts without batch-to-batch problems. This has made it possible to develop a monoclonal antibody-based sandwich ELISA that allows the detection and quantification of Lep d 2 in the environment using rLep d 2 as standard. The aims of this study were to develop the Lep d 2 assay, and to monitor Lep d 2 as well as Der p 1, Der f 1 and Der 2 allergen levels in dust samples from Gotlandic dairy farmers' households. Furthermore we wished to investigate whether exposure to these allergens is associated with sensitization, asthma and/or rhinoconjunctivitis.

Materials and methods

Subjects

The study population was composed of 393 Swedish farmers on the island of Gotland in the Baltic Sea who in 1996 had participated in a follow-up survey regarding allergic respiratory diseases [6]. All subjects had completed a questionnaire on symptoms from the upper and lower airways and attended a medical examination, which comprised a structured interview, skin prick tests (SPT)s and blood sampling for determination of IgE antibodies to common aeroallergens (Pharmacia CAP System Specific IgE FEIA; Pharmacia Diagnostics, Uppsala, Sweden).

Based on the results from the questionnaire and the medical examination, asthma was present in 71 subjects and rhinoconjunctivitis in 219 of the 393 farmers. Sensitization to *L. destructor* and *D. pteronyssinus* was recorded in 69 and 49 subjects, respectively. Sensitization was defined as at least a positive SPT (2+ and at least 3 mm) and/or a positive CAP FEIA (>0.35 kU/L). Thirty-three subjects were positive in CAP FEIA to rLep d 2 [15].

Dust sampling and extraction

Dust samples were collected from bed mattresses and living-room carpets. The farmers were instructed to use their own vacuum cleaners with a new dust bag, and to vacuum 1 m² for 2 min. A separate filter bag (Medeca AB, Uppsala, Sweden) was inserted into the inlet tube of the vacuum cleaner for each

sampling site. The subjects were advised not to vacuum their mattresses and carpets for 14 days prior to collection of dust. After collection, the filter bags were sealed in plastic bags and stored at –20 °C until extracted. The dust samples were then prepared as described earlier [17]. Briefly, they were sieved through a 300-µm mesh screen and 50–100 mg of fine dust was extracted (1/20 w/v) in phosphate-buffered saline (PBS) containing 0.1% Tween 20. The extracts were then centrifuged and the supernatants collected and stored at –20 °C until analysis of the allergen content.

At least one sample from either mattresses or carpets from 393 households was available for Der p 1, Der f 1, Der 2 and Lep d 2 analysis. The number of available dust samples for analysis from mattresses and carpets, respectively, is presented in Table 1.

Development of a monoclonal antibody-based Lep d 2 sandwich ELISA

Antigens and allergens The recombinant allergen Lep d 2.01 (rLep d 2) was used as the immunizing antigen in the production of monoclonal antibodies (mAbs). Lep d 2 has previously been cloned [13] and expressed as a 6-histidine tagged recombinant protein and was shown to have the same IgE reactivity as the native Lep d 2 [14]. For this study the allergen was expressed without a His-tag using the pET expression system (Novagen Inc., Madison, WI, USA). The rLep d 2 was then purified from solubilized inclusion bodies by FPLC anion chromatography on a High Q column (Econopac: Bio-Rad, Richmond, CA, USA) with a salt gradient of 0–0.5 M NaCl in 20 mM Tris-HCl pH 8.5. The allergen was eluted at a concentration of approximately 0.25 M NaCl, dialysed against PBS and stored in aliquots at –20 °C until used.

The specificity of the mAbs produced was checked against different mite species. Sieved whole mite cultures from *L. destructor*, *Tyrophagus putrescentiae*, *Acarus siro*, *Glycyphagus domesticus* and *D. pteronyssinus* (Allergon AB, Ängelholm, Sweden) were extracted in 0.15 M NaCl, 0.02% NaN₃ at 1/10 w/v overnight at room temperature (20 °C), centrifuged and filtered through a 0.8-µm Millex-HA filter (Millipore, Bedford, MA, USA). The extracts were stored at –20 °C until used.

Production, characterization and labelling of Lep d 2 mAbs

Ten female A/J mice were immunized for production

Table 1. Concentrations of Lep d 2, Der p 1, Der f 1 and Der 2 in mattresses and carpets in 393 houses

	Lep d 2		Der p 1†		Der f 1†		Der 2†	
	Mattress	Carpet	Mattress	Carpet	Mattress	Carpet	Mattress	Carpet
No. of samples	344	356	346	355	326	352	327	353
Median (µg/g)	< 0.09*	< 0.09*	0.094	< 0.055*	< 0.055*	< 0.055*	< 0.055*	< 0.055*
Min–Max (µg/g)	< 0.09–1.41	< 0.09–1.72	0.057–139	< 0.055–15.7	< 0.055–24.6	< 0.055–6.07	< 0.055–24.3	< 0.055–12.6
Samples <i>n</i> (%) with Lep d 2 (µg/g):								
< 0.090	288 (83.7)	323 (90.7)						
0.09 – < 2	56 (16.3)	33 (9.3)						
Samples <i>n</i> (%) with Der p, Der f (µg/g):								
< 0.055			150 (43.4)	225 (63.4)	201 (61.7)	286 (81.3)	170 (52.0)	241 (68.3)
0.055 – < 2			155 (44.8)	119 (33.5)	111 (34.0)	66 (18.7)	138 (42.2)	107 (30.3)
2.0 – < 10			25 (7.2)	7 (2.0)	10 (3.1)		13 (4.0)	4 (1.1)
> 10.0			16 (4.6)	4 (1.1)	4 (1.2)		6 (1.8)	1 (0.3)

†Significant difference between the levels of allergen collected in mattresses and carpets, *P* < 0.0001. *Median values below detection limit.

of antibodies. The mice were primed with 50 µg rLep d 2 in Freund's complete adjuvant (Difco, Boule Nordic AB, Huddinge, Sweden) by intraperitoneal injections, and were boosted three times at 2–4 week intervals with 10–20 µg in Freund's incomplete adjuvant. The last prefusion dose (20 µg, in PBS) was given intrasplenically [18]. The mouse with the highest antibody titre was killed and the spleen cells were mixed with Sp2/0 myeloma cells using standard protocols. Two weeks after the fusion the hybridoma supernatants were screened for antibody reactivity against rLep d 2 and *L. destructor* extract by direct ELISA. Only mAbs of IgG class were detected by using a goat anti-mouse IgG conjugate (MabTech, Stockholm, Sweden) in the screening test. Serum from the mouse from which the mAbs were obtained was used as a positive control and the culture supernatant from the myeloma Sp2/0 was used as negative control. Clones reacting to both rLep d 2 and *L. destructor* extract were subcloned by limiting dilution and tested for cross-reactivity to other mite species (*T. putrescentiae*, *A. siro*, *G. domesticus* and *D. pteronyssinus*) by ELISA.

The subclass of the mAbs was determined by ELISA using anti-mouse subclass specific conjugate (Southern Biotechnology Associates, Birmingham, AL, USA). Purification from the hybridoma culture supernatants was then performed by affinity chromatography using protein A Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The protein concentration of the purified mAbs was determined using Pierce bicinchoninic acid (BCA) reagent (Pierce Chemical Company, Rockford, IL, USA).

Purified mAbs were labelled with sulfo-NHS-LC Biotin (Pierce) according to the manual from the supplier. Excess of biotin was separated from the labelled antibodies by size-exclusion chromatography on a NAP-10 column (Amersham). The biotin conjugate was stored in PBS with 0.02% NaN₃ at 4 °C.

Lep d 2 ELISA Microtitre 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 µL of anti-Lep d 2 mAb 10B9 at 10 µg/mL in carbonate buffer pH 9.6. All steps thereafter were performed using 100 µL per well at room temperature. The coated wells were blocked for 1 h with PBS containing 0.05% Tween 20 and 0.2% BSA. After washing, serial diluted rLep d 2 standard (2700–3.7 ng/mL) and dust samples diluted 1.25 times, were added in duplicates and incubated overnight. The standard and dust samples were heated to 100 °C for 2 min before dilution. The wells were then washed and sequentially incubated for 2 h with biotin-labelled Lep d 2 mAb 7C6 and for 1 h with streptavidine/alkaline-phosphatase conjugate (DAKO, Glostrup, Denmark) with washing in between. Finally, the wells were incubated with alkaline phosphatase (ALP)-substrate (p-nitrophenyl phosphatase disodium (Sigma Diagn. St Louis, MO, USA) in the dark until the highest standard concentration reached an absorbance of 2.0–2.2 at 405 nm. The Lep d 2 content of the samples was obtained by interpolating in the rLep d 2 standard curve.

The intra- and inter-assay coefficients were calculated by analysing five replicates of the same sample in eight independent assays.

To investigate the correlation between the levels of Lep d 2 and the number of mites in a group of samples collected from barns, the Lep d 2 content was measured in 28 hay dust samples

which had earlier been microscopically investigated for their content of *L. destructor* [10].

Der 1 and Der 2 ELISA

The allergen contents of Der p 1, Der f 1 and Der 2 were determined by a two-site ELISA using monoclonal antibodies. The assays were performed according to the protocols from Indoor Biotechnology (Charlottesville, VA, USA) with the exception that peroxidase-labelled conjugates were substituted with ALP-labelled streptavidine (DAKO).

The extracts were assayed in duplicate at 2.5-fold dilution and reanalysed if they differed by more than 10%. Allergen concentrations were expressed as µg/g of fine dust. Dust samples with Der 1 and Der 2 levels below the detection limit (0.055 µg/g) were assigned a value of 0.054 µg/g for statistical analysis.

Statistical methods

Due to the skewed distribution of the allergen levels, the results are reported as median, minimum and maximum values. In order to statistically analyse data by a non-parametric method, concentrations lower than the detection level (0.090 µg/g for the Lep d 2 assay and 0.055 µg/g for the Der p 1, Der f 1 and Der 2 assays), were assigned values of 0.089 and 0.054 µg/g, respectively. To determine the levels of significance between two groups, the Mann–Whitney *U*-test was used. The Spearman rank correlation was used to investigate the correlation between levels of Lep d 2 and the number of mites. Finally, logistic regression analysis was used for the calculation of associations between allergen levels and sensitization to mites, allergen levels and symptoms, sensitization to mites and symptoms, and positive CAP analysis to rLep d 2 and symptoms. Independent variables were categorized into positive (above the detection limit of the assay) or negative (below the detection limit of the assay) allergen and IgE values. The odds ratio (OR) was expressed with corresponding 95% confidence interval (CI). If the CI did not include 1.0, the results were considered statistically significant. The conventional level of < 5% was set for statistical significance.

Results

Characterization of mAbs

Fourteen mAbs of IgG class were obtained from a single fusion of spleen cells from a mouse immunized with rLep d 2 and SP2/0

Table 2. Specificity of sandwich ELISA for quantification of Lep d 2

Sample	OD405
rLep d 2	1.858
<i>Lepidoglyphus destructor</i>	1.956
<i>Tyrophagus putrescentiae</i>	0.060
<i>Acarus siro</i>	0.052
<i>Glycyphagus domesticus</i>	0.053
<i>Dermatophagoides pteronyssinus</i>	0.051
Blank	0.063

rLep d 2 was tested in sandwich ELISA at 1 µg/mL and the mite extracts at 500 µg/mL using mAb 10B9 as capture antibody and mAb 7C6 as tracer.

myeloma cells. Five of these antibodies, which were of subclass IgG2b, also reacted to *L. destructor* extract and did not show cross-reactivity to other mite species (*T. putrescentiae*, *A. siro*, *G. domesticus* and *D. pteronyssinus*). The 25 possible combinations of the five selected mAbs were analysed using serial dilutions of rLep d 2 in a two-site ELISA. The best results were obtained with mAb 10B9 as the capture antibody and the biotin-labelled mAb 7C6 as the tracer. The specificity of the ELISA is shown in Table 2.

ELISA for quantification of Lep d 2

rLep d 2 was serially diluted from 2700 to 3.7 ng/mL and used as the standard curve (Fig. 1). The detection limit of the assay was 3.7 ng/mL, which corresponds to 0.09 µg/g of dust.

The Lep d 2 assay was highly reproducible. The intra- and inter-assay coefficient, based on five replicates analysed at eight different occasions, was 5% and 13%, respectively.

Lep d 2 content and correlation with mite count

The levels of Lep d 2 were correlated to the number of mites in 28 hay dust samples collected from barns (Fig. 2). The concentration of Lep d 2 ranged from 0.09 µg/g to 36.5 µg/g of dust and the number of *L. destructor* mites varied from 430 to 195 400 counts. The results show a strong correlation between the levels of Lep d 2 and the number of mites ($r_s: 0.7$, $P = 0.0002$).

Allergen levels in farmers' houses

Lep d 2 levels Lep d 2 was detected in 19.8% of the farmers' houses (78/393). The distribution and the levels of Lep d 2 in mattresses and carpets are presented in Table 1. Lep d 2 was

more frequent in mattresses than in carpets but there was no significant difference between the levels. The median value in both mattresses and carpets was under the detection level of the Lep d 2 assay, 0.09 µg/g, but still 16.3 and 9.3%, respectively, of the samples were positive. The levels of Lep d 2 in mattresses and carpets were in the same range: none above 2 µg/g of dust.

Der p 1, Der f 1 and Der 2 levels As expected, Der p 1 was most frequently detected of the three different *Dermatophagoides* allergens. It was found in 59% of the houses (231/393), followed by Der 2, detected in 49.9% (196/393) and Der f 1 in 39.9% (157/393). Der p 1 was also found in higher levels in both mattresses and carpets than the other two allergens (Table 1). A median value above the detection limit (0.055 µg/g) was only obtained for Der p 1 in samples from mattresses, where it was found to be 0.094 µg/g. All three allergens: Der p 1, Der f 1 and Der 2, were found in significantly higher levels in mattresses than in carpets ($P < 0.0001$). Der p 1 levels above 2 µg/g of dust were detected in 11.8% of the mattresses but only in 3.1% of the carpets. Concentrations above 2 µg/g were more frequent in mattresses than in carpets also for Der 2 and Der f 1 (Table 1).

Association of mite allergen exposure, sensitization, and symptoms

The association of allergen levels in dust samples and sensitization to mites and symptoms was calculated with logistic regression analysis. The Lep d 2 values (> 0.09 µg/g of dust) were neither associated with sensitization to *L. destructor*, nor with sensitization to rLep d 2. In addition, the Lep d 2 values were

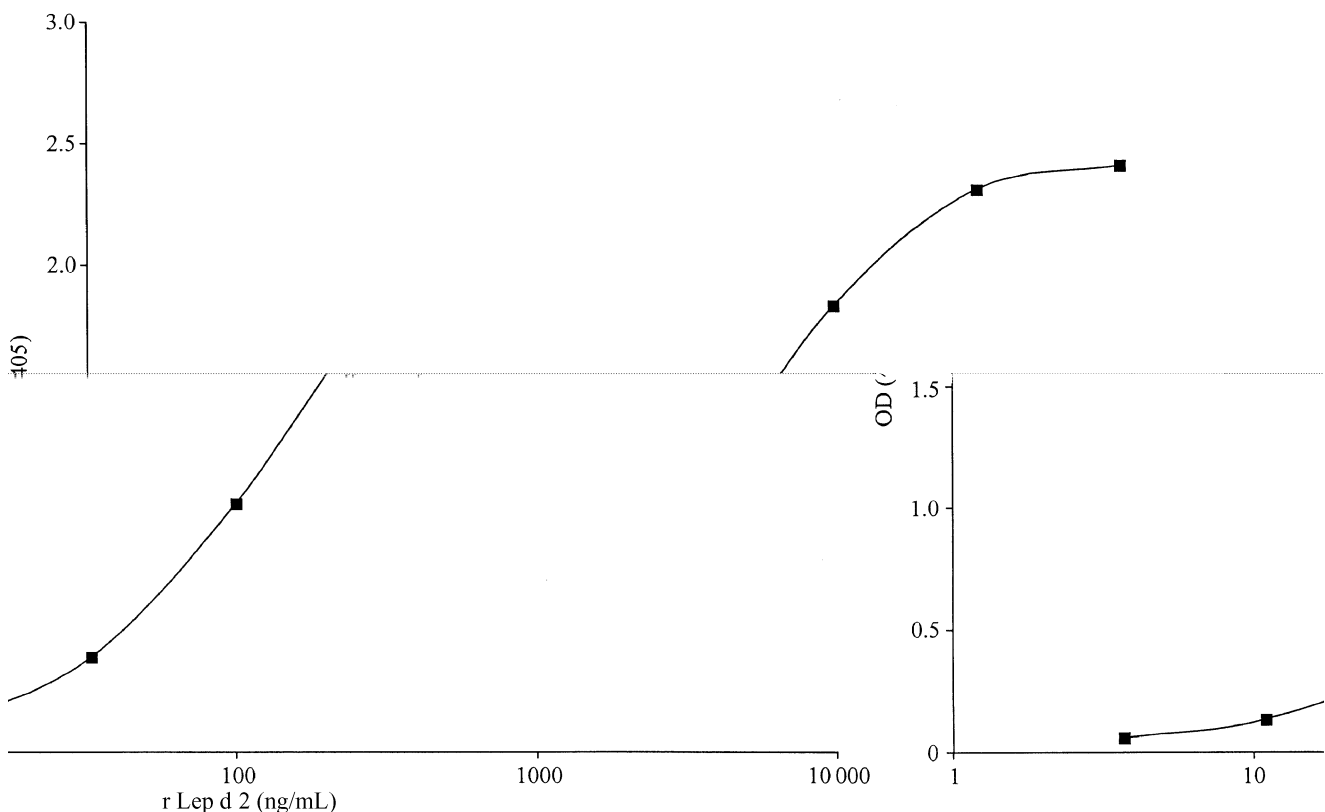


Fig. 1. Standard curve for the mAb-based Lep d 2 ELISA using recombinant Lep d 2.

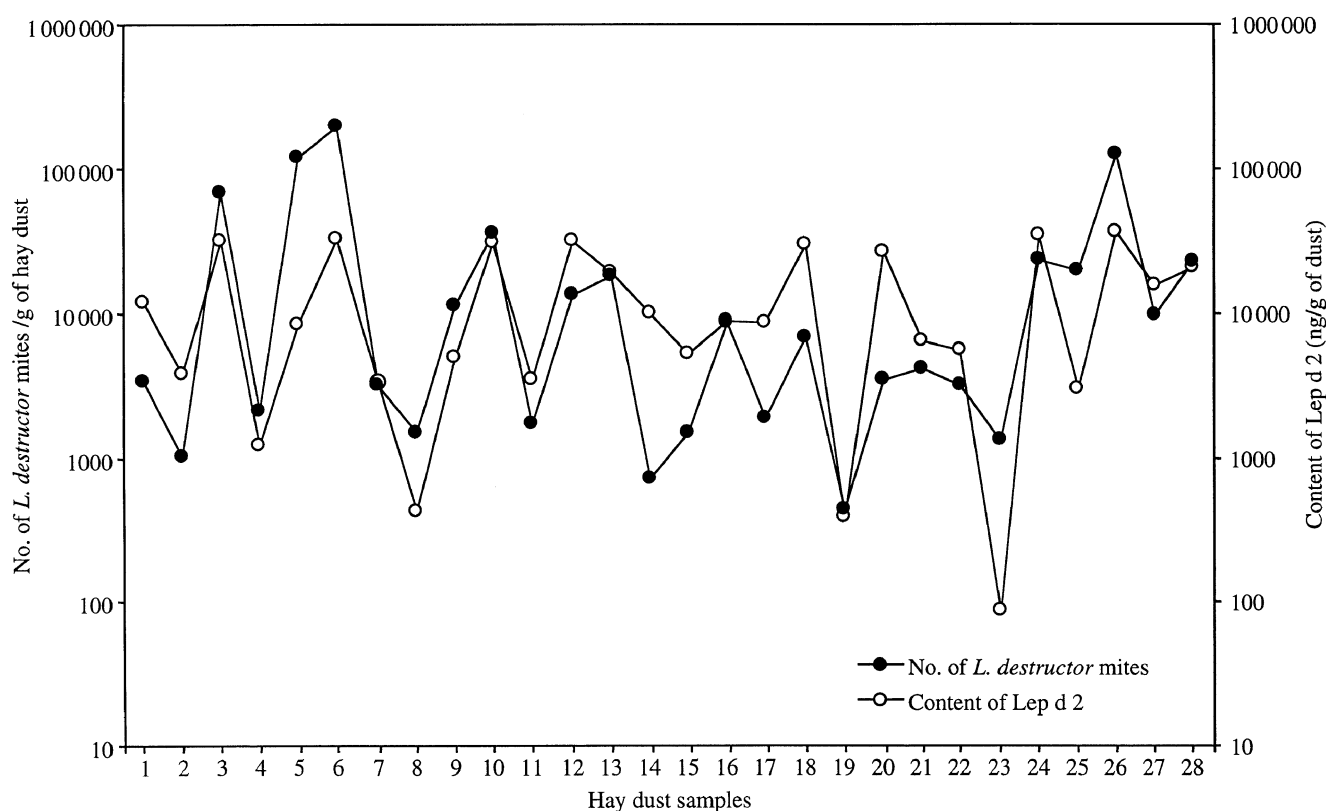


Fig. 2. Correlation between levels of Lep d 2 measured in mAb-based Lep d 2 ELISA and number of mites counted in 28 hay dust samples.

not associated with symptoms. Likewise, the Der p 1, Der f 1 and Der 2 levels ($> 0.055 \mu\text{g/g}$ of dust) lacked association with either sensitization to *D. pteronyssinus* or symptoms.

Furthermore, sensitization to *L. destructor* did not show association with symptoms; but, a strong relationship could be seen between IgE values to rLep d 2 on the one hand, and asthma (OR: 10.4, CI: 2.8–38.6, $P < 0.001$) and rhinoconjunctivitis (OR: 7.5, CI: 2.2–25.3, $P < 0.001$) on the other. Sensitization to *D. pteronyssinus* had however, an influence on both asthma (OR: 13.7, CI: 3.6–51.2, $P < 0.001$) and rhinoconjunctivitis (OR: 5.7, CI: 1.6–20.1, $P < 0.001$).

Discussion

The major allergen of *L. destructor*, Lep d 2, was detected in nearly 20% of the 393 homes, even though the median level was below the detection limit of the assay. A higher frequency of Lep d 2 was observed in samples obtained from mattresses than from carpets, but the levels were in the same range from both sampling sites. Radon and colleagues, who in a recent study investigated the indoor allergen exposure among pig farmers, detected Lep d 2 in 63% of dust samples from farmers' mattresses, but the major reservoir was in the grain mill [12]. Their higher figure of detectable Lep d 2 levels was probably due to the existence of a transit area, a room located between the confinement house and the kitchen. They found that the concentration of Lep d 2 in this area was related to the Lep d 2 concentrations in farmers' beds. Nevertheless, the Lep d 2 levels they recorded were in the same order to those we observed.

Echechipía and colleagues [19], who monitored the indoor environment in a Spanish urban population, recently reported the same frequency of occurrence of Lep d 2 as we have, and a magnitude close to the one we report here. Furthermore, a study from Portugal, which focused on indoor mite allergen levels from homes of patients with respiratory allergy, reported Lep d 2 in 44% of the 59 homes examined. However, the Lep d 2 allergen was always present in very low concentrations ($< 1 \mu\text{g/g}$ dust) in spite of high outdoor humidity and numerous homes with signs of dampness [20]. As regards Sweden, Warner and colleagues, who investigated mite infestation in various sites in Swedish households, demonstrated that the predominant sites of storage mite occurrence were kitchens and bathrooms, even though these mite species could occur in bedrooms and livingrooms [8]. This indicates that we might have observed higher Lep d 2 levels if sites other than the livingroom (carpets) and bedrooms (mattresses) had been investigated.

Der p 1 was found to be the predominant mite allergen in the 393 farmers' homes examined. Nearly 12% of samples from mattresses had Der p 1 levels above $2 \mu\text{g}$ whereas Der f 1 and Der 2 were found in 4% and 6%, respectively. Radon and colleagues [12], also recorded high Der p 1 allergen levels in farmers' mattresses, but their levels were higher than the ones we observed. Furthermore, Iversen and colleagues have likewise reported the presence of high numbers of *Dermatophagoides* mites in the mattresses of farmers [5].

We could not show any association between Lep d 2 levels and sensitization to *L. destructor*, which is probably owing to the low Lep d 2 exposure in rural homes. The major reservoir of *L. destructor* seems to be the workplace of the farming

environment, where we, as well as others, have demonstrated very high amounts of *L. destructor* [5,10,21]. Thus, occupational exposure to *L. destructor* is more significant in terms of sensitization than domestic exposure. As regards the *Dermatophagoides* allergens, we likewise did not obtain a significant association between allergen levels of either Der p 1 or Der 2, and sensitization to *D. pteronyssinus*. However, we found that the IgE reactivity against rLep d 2 as well as sensitization to *D. pteronyssinus* was associated with asthma and rhinoconjunctivitis. This is in accordance with other reports where sensitization to mites has been shown to be a risk factor for asthma and rhinoconjunctivitis [1,22].

The ELISA developed in this study for quantifying Lep d 2 in the environment utilizes a recombinant allergen, rLep d 2, as standard. This is in line with the intention of WHO/IUIS Allergen Standardization Committee [23] who is starting a program to produce purified natural and recombinant allergens as primary standards for immunoassays. In fact, there are already assays for the assessment of allergen exposure, which use recombinant allergens as standards [23,24]. There are several advantages to using recombinant allergens as standard. The purity of the allergen offers a more correct protein determination than what is possible with whole extracts that have been calibrated to a known concentration. Recombinant allergens can be produced in large amounts and problems with assay variability due to quantitative differences between allergen standards can be avoided. In the Lep d 2 assay we used mAbs, which were obtained after immunization with rLep d 2. The mAbs are highly specific, as none of them binds to allergens from other mite species that have earlier been shown to cross-react with *L. destructor* [25,26]. The sensitivity of the assay is in the same order as most ELISAs measuring indoor allergens [23] and the intra- and inter-assay variation of the assay was 5% and 12%, respectively, results that are in agreement with those of other assays for quantification of indoor allergens [23]. A strong correlation between the levels of Lep d 2 and number of *L. destructor* mites was achieved in samples with known number of mites. Taken together, the Lep d 2 assay is sensitive and specific and discriminates between relatively high Lep d 2 levels in barn dust and low levels in dust from farm-houses.

In conclusion, even though we found that allergens from the *Dermatophagoides* species are dominating in rural households, we detected Lep d 2 in nearly 20% of farmers' homes. The major *L. destructor* allergen, Lep d 2, which has to be considered when assessing mite allergen exposure in farming surroundings, can now be measured with a specific ELISA suitable for quantifying Lep d 2 in dust samples from the indoor as well as the outdoor environment.

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