

Species-specific measurement of the second group of *Dermatophagoides* mite allergens, Der p 2 and Der f 2, using a monoclonal antibody-based ELISA

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Summary

Background The group 2 *Dermatophagoides* mite allergens, Der p 2 and Der f 2, were known to be highly crossreactive, and previous assays to measure Der p 2 and Der f 2 were not species-specific.

Objective The aim of this study was to develop a monoclonal antibody-based ELISA (MoAb-ELISA) to species-specifically measure Der p 2 and Der f 2.

Methods The MoAb-ELISA for Der p 2 and Der f 2 was performed using species-specific MoAbs for Der p 2 and Der f 2 and a biotinylated second MoAb which recognized a common epitope on both Der p 2 and Der f 2.

Results The assay was highly species-specific, reproducible and sensitive. Thirty-two house dust samples were assayed by the MoAb-ELISA for Der p 2 and Der f 2 and by a previously reported radioimmunoassay for Der 2 with rabbit anti-Der 2 antibodies. The summed values for Der p 2 and Der f 2 by the MoAb-ELISA demonstrated a good correlation with the Der 2 values using the radioimmunoassay ($r=0.978$). Furthermore, the proportion of the Der p 2 level in the total Der 2 level (Der p 2 divided by Der p 2 plus Der f 2) correlated well with that of the *D. pteronyssinus* mite number to the total *Dermatophagoides* mite number identified by species ($r=0.970$).

Conclusion The MoAb-ELISA for Der p 2 and Der f 2, as well as that for Der p 1 and Der f 1, will be useful for the standardization of mite extracts and for the assessments of mite allergen exposure.

Keywords: *Dermatophagoides* mite, Der p 2/Der f 2, monoclonal antibody, ELISA, species-specificity

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Introduction

Immediate hypersensitivity to house dust allergens is an important cause of asthma, rhinitis and atopic dermatitis. It has been established that *Dermatophagoides* mites, *D. pteronyssinus* and *D. farinae*, are the major source of house dust allergens [1,2]. Two groups of *Dermatophagoides* mite major allergens, Der 1 (group 1) and Der 2

(group 2) allergens, have now been characterized and cloned [1–4].

The measurement of specific major allergens by an immunochemical assay has principal applications for the standardization of allergenic extracts and for the assessment of environmental allergen exposure [5–8]. Although there have been a variety of radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs) to species-specifically measure Der p 1 and Der f 1 [9–13], previous assays developed for the Der 2 allergens were not species-specific [12,14,15].

The present study describes the development of a

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monoclonal antibody-based ELISA (MoAb-ELISA) to species-specifically measure Der p 2 and Der f 2. This assay, as well as the MoAb-ELISA for Der p 1 and Der f 1 [13], was used to examine the relationship among the Der 1 and Der 2 allergen levels and mite numbers in 32 house dust samples.

Materials and methods

Purified mite allergens

The mite allergens, Der p 2 and Der f 2, were purified from the whole cultures of *D. pteronyssinus* and *D. farinae* as previously described [4]. The concentrations of the purified allergens were determined from their extinction coefficient values, $E_{1\text{ cm}}^{1\%}$ at 280 nm = 6.6 for Der p 2 and 5.8 for Der f 2 [2,4].

MoAbs to Der p 2 and Der f 2

Mouse MoAbs to Der p 2 and Der f 2 were obtained from hybridoma cell lines prepared by the fusion of spleen cells from A/J mice immunized with purified Der p 2 or Der f 2 in complete Freund's adjuvant with P3X63-Ag8.653 myeloma cells. Antibody producing hybridoma cells were screened by an ELISA using Der p 2 or Der f 2 which had been immobilized on microtitre plate wells via rabbit anti-Der 2 antibodies [12] and peroxidase conjugated goat anti-mouse IgG. Antibody-positive hybridoma were cloned at least twice by limiting dilution. Ascites was produced by injecting 10^7 cells ip into CAF-1 mice which had been previously treated with pristane. MoAbs were purified from each ascites by affinity chromatography on a protein A column (Ampure PA kit, Amersham Japan, Tokyo, Japan).

Biotinylation of MoAbs

One milligram of MoAb in 1 mL of 0.1 M NaHCO_3 was mixed with 0.1 mg of *N*-hydroxysuccinimidobiotin (Sigma Chemical Co., St Louis, MO, USA) in 0.1 mL of dimethylformamide and incubated at 25°C for 4 h. Biotinylated MoAb was separated from unreacted biotin through a Sephadex G-50 (Pharmacia Biochemistry, Uppsala, Sweden) column.

MoAb-ELISA for Der p 2 and Der f 2

Polystyrene microtitre plates (MaxiSorp, Nunc, Denmark) were coated with 500 ng of P2A24 (anti-Der p 2 MoAb) or F2A41 (anti-Der f 2 MoAb) in 100 μL of 0.1 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. The plates were washed three times with phosphate

buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) and incubated with 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. After washing twice with PBS-T, 100 μL of Der p 2 or Der f 2 standard solution (64–0.5 ng/mL), or dilutions of mite or house dust extracts in PBS-T-BSA were added to the wells and incubated at 37°C for 2 h. The plates were washed five times with PBS-T and then incubated with 100 ng of biotinylated anti-Der p 2 MoAb P2A91 which was crossreactive to both Der p 2 and Der f 2 in 100 μL of PBS-T-BSA at 37°C for 2 h. After washing five times with PBS-T, the plates were incubated with 100 μL of streptavidin- β -D-galactosidase conjugate (Boehringer Mannheim, Germany) diluted 1:1000 in PBS-T-BSA at 37°C for 1 h and then washed five times. Finally, 100 μL of 5 mM *o*-nitrophenyl- β -D-galactopyranoside in 0.02 M sodium phosphate containing 0.1 M NaCl, 1 mM MgCl_2 , 0.2% BSA and 0.05 M NaN_3 , pH 7.2, was added to the wells and incubated at 37°C for 60 min. After the enzyme reaction was stopped with 100 μL of 0.1 M Na_2CO_3 , the absorbance at 415 nm was measured using a BIO-RAD 3550 microplate reader (Bio-Rad Laboratories, Richmond, CA, USA).

Der p 1 and Der f 1 in mite and house dust extracts were measured by the MoAb-ELISA as previously described [13].

Mite extracts

The preparation of the mite reference extracts, 92-Dp and 92-Df, has been described elsewhere [13]. The WHO international standard for house dust mite (*D. pteronyssinus*) extract (NIBSC 82/518) was obtained from the National Institute for Biological Standards and Control, London, UK. The CBER standard mite extracts, E4-Dp and E5-Df, were provided by Dr Y. DeVries, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA. Commercial mite extracts were obtained from Miles Inc., Elkhart, IN, USA and Torii & Co. Ltd, Tokyo, Japan.

House dust samples

Thirty-two dust samples were obtained from household vacuum cleaner bags from different homes. The fine dust was collected and extracted as previously described [12].

Mite counts and identification of mite species were performed according to the method of Miyamoto and Ouchi [16]. Briefly, $\frac{1}{2}$ g of the fine dust was mixed in 50 mL of Darling's solution (1:1 mixture of glycerol and saturated NaCl) and 2 mL of 0.2% sodium dodecyl sulfate with stirring for 10 min. After centrifugation, the supernatant with floating mite bodies was filtered using a Buchner funnel. The sediment was again mixed in

Darling's solution and treated in the same manner. The resultant two filter papers were examined under a dissecting microscope. Observed mite bodies were mounted and identified under a compound microscope.

Results

Establishment of MoAb-ELISA for Der p 2 and Der f 2

Five MoAbs to Der p 2 and 11 MoAbs to Der f 2 were obtained from two fusions against Der p 2 and three fusions against Der f 2, respectively. From a total of 16 MoAbs, two MoAbs to Der p 2, P2A24 and P2A91, and a MoAb to Der f 2, F2A41, were selected for use in the MoAb-ELISA for Der p 2 and Der f 2. P2A24 and F2A41, which recognized a species-specified epitope on Der p 2 and Der f 2, respectively, were used as 'capture' antibodies on the solid phase, and P2A91, which was crossreactive to Der p 2 and Der f 2, was used as a biotinylated second antibody. Typical dose-response curves of the purified Der p 2 and Der f 2 are shown in Fig. 1. Both assays for Der p 2 and Der f 2 were sensitive (1 ng/mL) and highly species-specific. In the MoAb-ELISA for Der p 2, the dilution curve of *D. pteronyssinus* reference extract, 92-Dp, was parallel to the Der p 2 standard curve, while no significant binding was seen with the purified Der f 2 and the *D. farinae* reference extract, 92-Df (Fig. 1a). Similar but reversed results were obtained in the MoAb-ELISA for Der f 2 (Fig. 1b). Dilution curves for house dust extracts were parallel to the standard curves in both assays (data not shown).

The intra-assay and interassay variations of the ELISA were examined using house dust extracts over the working ranges of each assay in three quadruplicate runs. The mean intraassay coefficients of variations of the quadruplicate measurements for the Der p 2 and Der f 2 assays were 4.5 and 5.8%, respectively, and the mean interassay coefficients of variation for the assays were 5.9 and 5.8%, respectively.

Der 1 and Der 2 allergen levels in mite extracts

The reference extracts, 92-Dp and 92-Df, were found to contain 10.3 µg/mL of Der p 2 and 10.7 µg/mL of Der f 2, respectively, when the purified Der p 2 and Der f 2 preparations were used as the primary standards. The levels of Der p 2 and Der f 2, as well as those of Der p 1 and Der f 1, in several *D. pteronyssinus* and *D. farinae* extracts including the WHO/IUIS international reference preparations of *D. pteronyssinus* and the CBER standard mite extracts, E4-Dp and E5-Df, were also measured (Table 1).

Der 1 and Der 2 allergen levels in house dust samples

The MoAb-ELISA for Der p 2 and Der f 2 was used to measure the Der 2 allergen levels in 32 house dust samples and the results were compared with those obtained by the RIA with rabbit anti-Der 2 antibodies [12]. The summed values for Der p 2 and Der f 2 determined by the MoAb-ELISA demonstrated a good correlation with the Der 2 values determined by the radioimmunoassay (RIA) (Fig. 2).

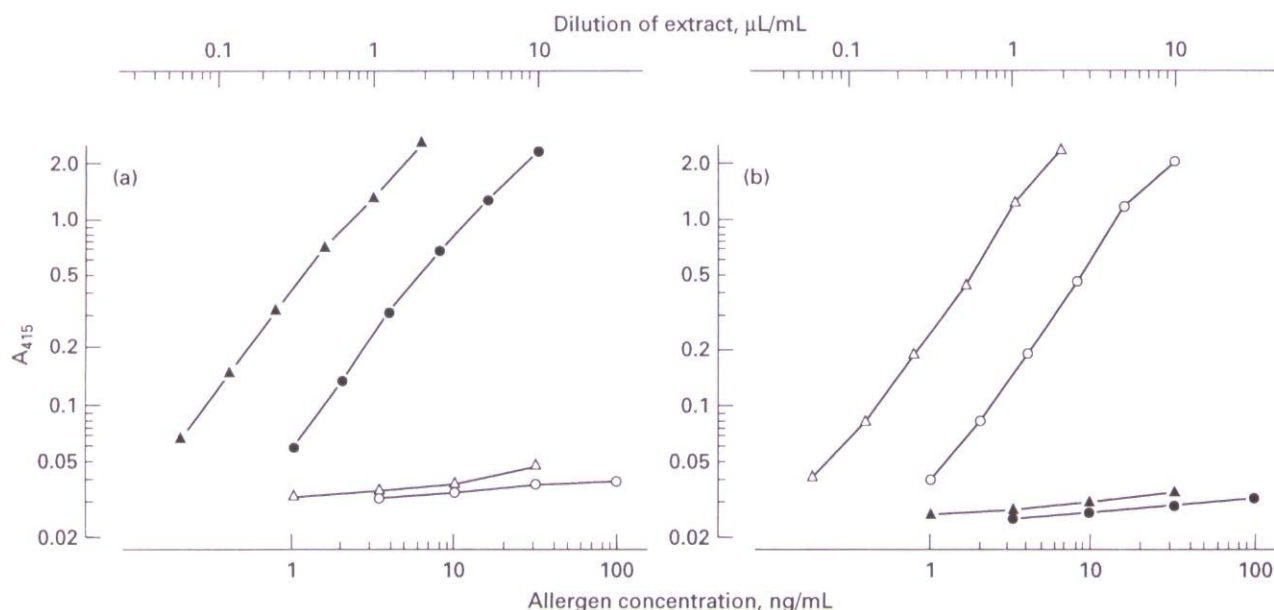


Fig. 1. Dose-dependent curves of the purified Der p 2 (●), and Der f 2 (○) and dilution curves of *D. pteronyssinus* and *D. farinae* extracts, 92-Dp (▲) and 92-Df (△), in the MoAb-ELISA for Der p 2 (a) and Der f 2 (b).

Table 1. Der 1 and Der 2 levels in *Dermatophagoides* mite extracts

Extract	Potency	Der 1	Der 2	Der1:Der2 ratio
		$\mu\text{g/mL}$		
<i>D. pteronyssinus</i>				
92- <i>Dp</i> *	—	10.1	10.3	1.0
E4- <i>Dp</i> †	—	13.3	11.5	1.2
<i>Dp</i> -IS‡	100 000 IU/mL	4.4	0.28	15.7
A§	10 000 AU/mL	16.9	14.7	1.2
<i>D. farinae</i>				
92- <i>Df</i> *	—	10.0	10.7	0.9
E5- <i>Df</i> †	—	9.5	7.2	1.3
B§	10 000 AU/mL	10.9	6.6	1.7
C§	1:100 (w/v)	4.2	5.9	0.7

* Mite reference extracts prepared in our laboratory. † CBER/FDA standard extracts.

‡ WHO/IUIS International Standard *D. pteronyssinus* NIBSC 82/518. § Commercial extracts prepared by two different manufacturers.

The same house dust samples were assayed by the MoAb-ELISA for Der p 1 and Der f 1 and were also examined by microscopy for mite number and mite species. The proportion of the *D. pteronyssinus* mite number in the total *Dermatophagoides* mite number identified by species (percentage of *D. pteronyssinus*)

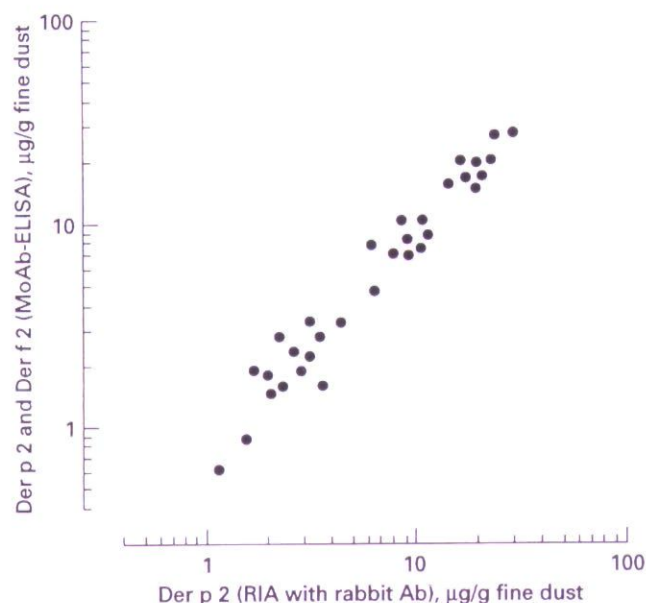


Fig. 2. Comparison of the Der 2 levels in 32 house dust samples as measured by the MoAb-ELISA for Der p 2 and Der f 2, and the RIA with rabbit anti-Der 2 antibodies. $r = 0.978$, $n = 32$.

was compared with that of the Der p 1 level in the total Der 1 level (percentage of Der p 1) and with that of the Der p 2 level in the total Der 2 level (percentage of Der p 2). A very good correlation was seen not only between the percentage of *D. pteronyssinus* and the percentage of Der p 1, but between the percentage of *D. pteronyssinus* and the percentage of Der p 2 (Fig. 3). A very good correlation was also seen between the percentage of Der p 1 and the percentage of Der p 2 ($r = 0.960$, data not shown).

As has already been reported [12,17], there was a very good correlation between the Der 1 and Der 2 allergen levels in the 32 dust samples ($r = 0.943$, data not shown). The mean ratio of Der 1 to Der 2 in the dust samples was nearly 1:1. A good correlation between the two groups of allergens in the dust samples was seen even in individual mite species, i.e. between the Der p 1 and Der p 2 levels and between the Der f 1 and Der f 2 levels (Fig. 4).

Discussion

The assay system developed in this study can species-specifically measure Der p 2 and Der f 2. Although there have been some previous assays for measuring the Der 2 allergens, they measured both Der p 2 and Der f 2 as 'Der 2' using crossreactive antibodies [12,14,15], or a single allergen, Der p 2 [18] or Der f 2 [19]. The Der 2 assay using crossreactive antibodies has the advantage that it can measure Der p 2 and Der f 2 in a single assay. However, it can not be used for the assessment of mite

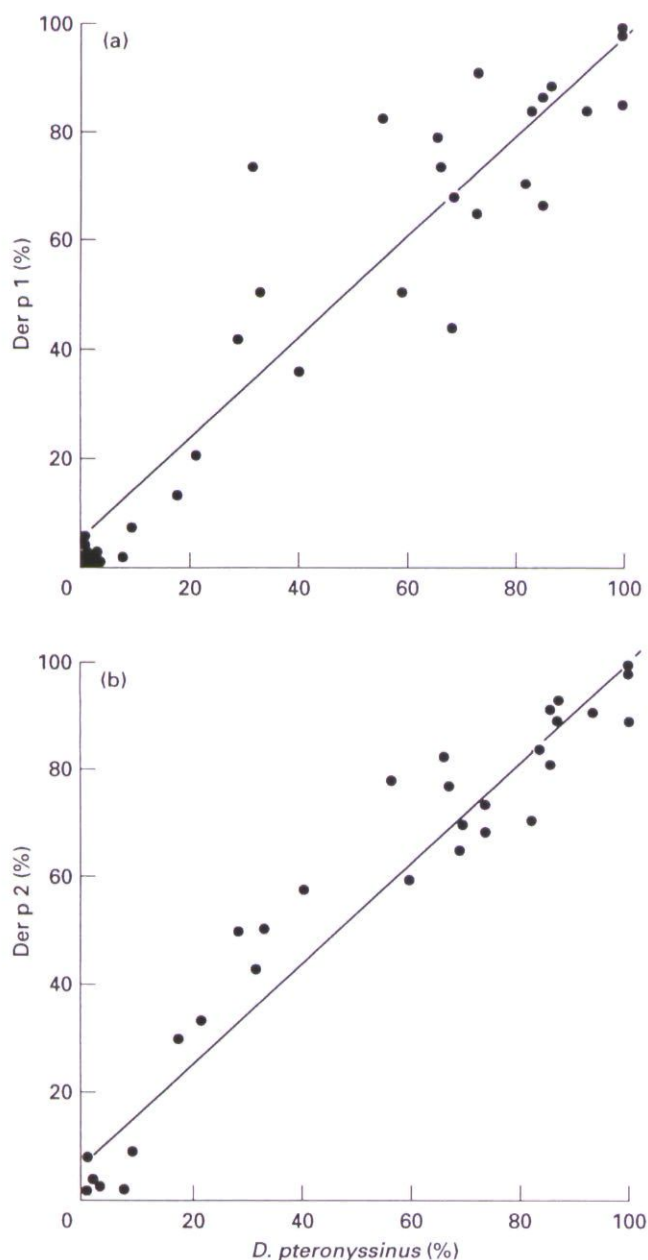


Fig. 3. Correlation between the proportions of the two mite species in 32 house dust samples identified by microscopy and those obtained with the MoAb-ELISAs for Der p 1 and Der f 1 (a), and for Der p 2 and Der f 2 (b). The percentage of *D. pteronyssinus* was calculated by dividing the *D. pteronyssinus* mite number by the *D. pteronyssinus* and *D. farinae* mite number times 100. The percentage of Der p 1 (Der p 2) was calculated by dividing the Der p 1 (Der p 2) level by the Der p 1 and Der f 1 (Der p 2 and Der f 2) level times 100. (a) $y = 0.92 \times +4.81$, $r = 0.936$, $n = 32$. (b) $y = 0.94 \times +6.98$, $r = 0.970$, $n = 32$.

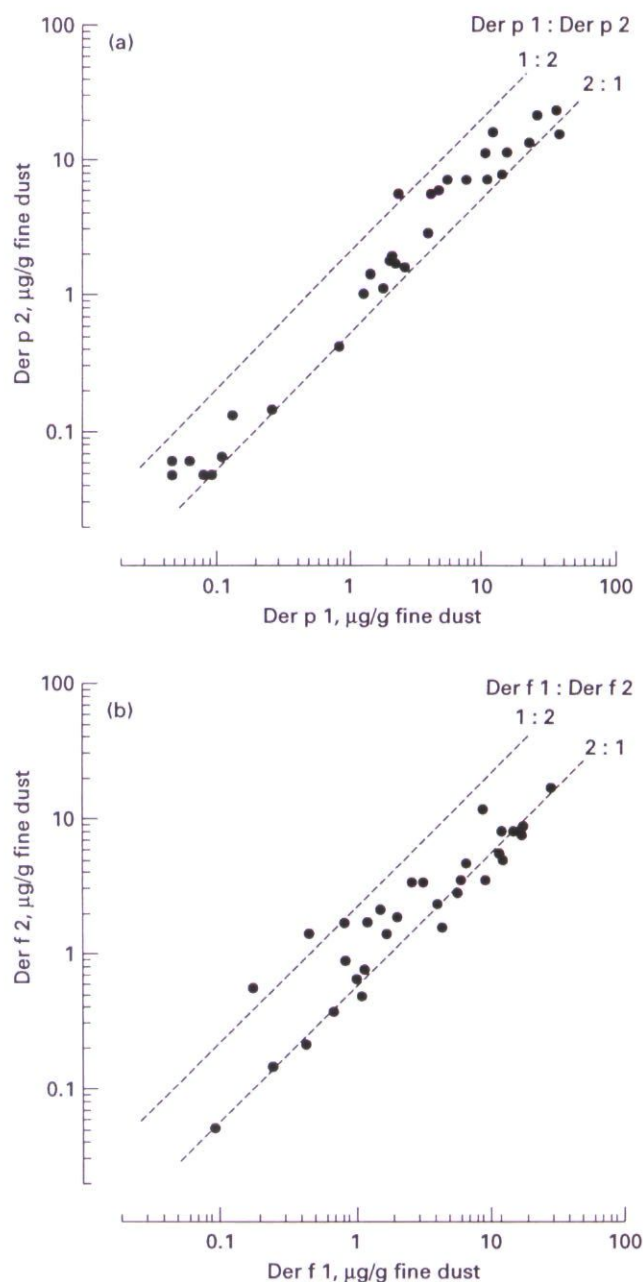


Fig. 4. Correlation between the Der p 1 and Der p 2 levels (a), and between the Der f 1 and Der f 2 levels (b) in 32 house dust samples. (a) $r = 0.985$, $n = 32$. (b) $r = 0.931$, $n = 32$.

allergen levels in individual houses in Japan or in the areas where the distribution of the two species of mites vary greatly from house to house (Fig. 3), unless the assay has comparative dose response curves for Der p 2 and Der f 2. If the assay has twice the reactivity to Der p 2 than to Der f 2, it will overestimate by two times the

Der 2 allergen levels of *D. pteronyssinus*-dominant house dust samples than those of *D. farinae*-dominant house dust samples. In the RIA previously reported [12], the comparative dose-response curves for Der p 2 and Der f 2 could be achieved by using the rabbit antibodies immunized with a 1:1 mixture of Der p 2 and Der f 2. The reactivity of Der p 2 and Der f 2 in MoAb-based Der 2 assays was tested using several pairs of crossreactive MoAbs produced in this study. The results indicated that the dose-response curves for Der p 2 and Der f 2 differed greatly, depending on the combination of two MoAbs used (unpublished observation). The choice of an appropriate combination of a MoAb for allergen capture and that for allergen detection will make it possible to measure Der p 2 and Der f 2 with an identical reactivity in a single assay.

The reference extracts, 92-*Dp* and 92-*Df*, were estimated to have the same levels of the Der 2 allergens, 10.3 µg/mL of Der p 2 and 10.7 µg/mL of Der f 2, respectively (Table 1). This result was consistent with our previous results that the 92-*Dp* and 92-*Df* had the same levels of Der p 1 and Der f 1 and that the total allergenic potency of both preparations as assessed by a histamine release assay was comparable [13]. Furthermore, our estimate of the Der p 2 level (11.5 µg/mL) in the CBER standard mite extract, E4-*Dp*, was in good agreement with that previously reported (10 µg/mL) [18].

In the production of MoAbs to Der p 2 and Der f 2, an ELISA screening for antibody producing hybridoma cells was performed using solid phase Der p 2 and Der f 2 which had been immobilized on microtitre plate wells not directly but via the rabbit anti-Der 2 antibody. All MoAbs selected by this screening had high affinity for the allergen and could be used both for allergen capture and for allergen detection in the sandwich ELISA for the Der 2 allergens.

The proportion of the two mite species in dust samples obtained with microscopical identification of mite species correlated very well with that obtained with the species-specific ELISAs for Der p 1 and Der f 1 and for Der p 2 and Der f 2 (Fig. 3). The result indicates that the species-specific ELISA for Der p 2 and Der f 2, as well as that for Der p 1 and Der f 1, can provide an accurate index of the distribution of *D. pteronyssinus* and *D. farinae* in individual dust samples.

The availability of species-specific ELISAs for both groups of the *Dermatophagoides* mite major allergens will provide more detailed and precise information on the allergenic features of mite extracts and house dust samples, and should be useful for the standardization of mite extracts, the assessments of mite allergen exposure and mite avoidance studies.

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