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Original Scientific Paper

DETERMINATION OF MITE ALLERGENS IN HOUSE DUST USING THE ENZYME IMMUNOASSAY

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The aim of this study was to determine the level of two major mite allergens *Dermatophagoides pteronyssinus* (Der p 1) and *Dermatophagoides farinae* (Der f 1) in 30 urban homes in Zagreb, Croatia, using the enzyme immunoassay with two monoclonal antibodies which has been established as the reference method for indoor allergen analysis. Dust samples were taken by vacuuming a carpeted area and collected on cellulose filters. The ranges of Der p 1 and Der f 1 were (0.1-12.5) $\mu g\ g^{-1}$ (median 0.32 $\mu g\ g^{-1}$) and (0.1-31.2) $\mu g\ g^{-1}$ (median 0.35 $\mu g\ g^{-1}$), respectively. Der p 1 and Der f 1 (>2 $\mu g\ g^{-1}$) associated with increased risk of sensitization to mite allergens were found in approximately 16 % homes for each allergen. The sum of allergen (Der p 1 + Der f 1) exceeded the lower threshold in 27 % of homes. Analytical evaluation of the ELISA assay showed satisfactory results for precision (intra-assay CV <6.9 %, inter-assay CV<13.3 %), accuracy (91 % to 93 %), and sensitivity (2 ng mL^{-1}).
The ELISA assay for the measurement of dust mite allergens demonstrated very good analytical characteristics for routine laboratory use, and will provide the essential basis for our future studies of various indoor allergens.

KEY WORDS: *Der f 1, Der p 1, ELISA, house dust mites, indoor allergens*

The indoor environment has been recognised as a common source of exposure to many allergens (1-4). However, mites are the most important source of allergens in settled house dust (5-7). The most prevalent mites found in the homes of Croatia (8, 9) and worldwide (6, 10) are *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*. Distribution of dust mites varies between geographic areas with different climate, and may be affected by housing characteristics (11, 12). Many studies investigated the relationship between the level of environmental exposure to dust mite with sensitisation and development of some allergic disease (2, 13). A threshold level of 2 μg and 10 μg of mite allergens per g of dust (lower and upper cut point) has been proposed for developing sensitisation to dust mite and for developing symptoms in already sensitised individuals, respectively (14, 15). Therefore, recommendations for measuring and monitoring mite allergen levels in the homes of mite-sensitive children and adults have been proposed in many countries (3, 16). Many studies have documented that removal of mite allergen from indoor environment may be of clinical benefit (17, 18).

The gold standard for measuring exposure to mite allergens, especially group 1 allergens in house dust, is the two-site monoclonal antibody (mAb) based enzyme immunoassay (ELISA) (19). Currently, this method provides the best index of allergen exposure in the settled dust (20). So far, ELISA has not taken root in Croatian laboratories for measuring mite allergen.

The aim of this study was to determine the level of mite allergens (Der p 1, Der f 1) in settled dust using the ELISA assay. Analytical evaluation of the method included intra- and inter-assay precision, accuracy, and sensitivity.

MATERIALS AND METHODS

Dust collection

Dust samples were obtained from 30 randomly selected homes in the Zagreb County during October 2006 and January 2007. In each home one dust sample was collected from the carpet of the main living area according to a standard protocol (14, 21). Samples were taken by vacuuming a carpeted area using standard vacuum cleaner adapter and cellulose filter (Heska AG, Freiburg, Switzerland). The dust was vacuumed until the filters were more than half full. The samples were stored at 4 °C and extracted the following day (11, 21).

Extraction procedure

A (100±5) mg dust samples (not sieved) were extracted with 2 mL of phosphate-buffer saline with Tween (PBS-T). Phosphate buffer (8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.20 g KH_2PO_4, Thimerosal 0.10 g in 1 L distilled water, pH 7.4) contained 0.05 % Tween 20 (3, 22). Thimerosal was added as preservative in the PBS-T. Extraction was done at room temperature for 2 h, under constant shaking. Dust extracts were centrifuged for 10 min at 3000 rpm. Supernatants were stored at -20 °C until analysed for allergen content.

Reagents

Allergen levels (Der p 1 and Der f 1) in the dust were measured using reagents for the ELISA assay purchased from Indoor Biotechnologies Ltd (Cardiff, UK). Both kits were supplied with double monoclonal antibodies (mAbs) and respective standard. As capture antibody for Der p1 and Der f1 assay we used monoclonal antibodies 5H8 and 6H8, respectively. Both assays used biotinylated monoclonal antibody 4C1 as detecting antibody. Assays for determination of Der p 1 and Der f 1 were standardised against reference standards defined by the World Health Organisation/International Union of Immunological Societies (WHO/IUIS). These standards (Der p 1, Der f 1) were declared to contain 2500 ng mL^{-1} of allergen. They were further diluted with 1 % bovine serum albumin (BSA) PBS-T (Sigma, USA) to obtain working solution in the concentration range of (1-125) ng mL^{-1} to construct the calibrating curve.

Dust extracts were diluted three, 30 times, and more if required using 1 % BSA-PBS-T.

Streptavidin-horseradish peroxidase (HRP) was used as detecting reagent, and a solution mixture of TMB (3, 3´,5´,5´-Tetramethylbenzidine) and peroxide were used as substrate (Adaltis, Italy). Reaction was stopped with 0.3 mol L^{-1} sulphuric acid. The reagents were kept at 4 °C.

The ELISA assay was performed at room temperature (19). All reagents were added to the microtiter wells in the volume of 0.1 mL.

ELISA protocol

Microplates (Nunc, Denmark) were coated with anti Der p1 or anti Der f1 monoclonal antibody (15 μL per 10 mL of 50 mmol L^{-1} sodium carbonate buffer, pH 9.6), covered with adhesive strip and incubated at room temperature overnight. Capture antibody was diluted immediately before use. After washing with PBS-T (three times), the plates were blocked with 1 % BSA-PBS-T (300 μL) for 30 min and washed. The plates were incubated with diluted samples and standards for 1 h. Then the wells were washed (three times) with PBS-T and treated with biotinylated antibody (10 μL per 10 mL of BSA-PBS-T) for 1 h and washed. All wells were then incubated with streptavidin-HRP for 30 min and washed. A substrate solution of TMB/peroxide was added and colour (blue) was developed for 15 min. The final step was to stop the reaction with stop solution which turned the solution yellow. The optical density was read after 10 min at 450 nm on PersonalLab (IASON, Graz, Austria) microplate analyser. Positive and negative controls were added to the respective wells. Measurements were done semi-automatically.

Each allergen sample was measured in duplicate. Concentrations of mite allergens were calculated from the calibrating curve prepared by dilution of standard stock solution. Results were calculated as microgram of allergen (Der p 1 or Der f 1) per gram of dust ($\mu g\ g^{-1}$).

Analytical evaluation of the method

Analytical evaluation included intra- and inter-assay precision, accuracy, and sensitivity. Intra-assay precision was established by multiple measuring (20x) of allergen concentrations in two dust mite extracts within one day. For Der p 1 and Der f 1 analysis, samples with low [(0.65±0.03) $\mu g\ g^{-1}$] and high allergen level [(26.4±1.84) $\mu g\ g^{-1}$] were used, respectively. The results were expressed as a coefficient of variation CV (%).

Inter-assay precision was determined by measuring the level of Der p 1 and Der f 1 during five days in four replicates. Extracts with low allergen level

(0.31±0.04) μg g^{-1} and (0.52±0.06) μg g^{-1} were used for both tests. The mean of daily measurements was used to calculate the between-day precision. The results were expressed as CV (%).

The accuracy of measurements was evaluated by the recovery test using two dust extracts with low and high allergen level according to the standard procedure. Samples were spiked with known concentrations of Der p 1 or Der f 1 and assayed. The results were expressed as the percent of expected value.

The sensitivity of the assay is defined by reliable discrimination from the zero standards using the mean absorbance of 20 replicates + three standard deviations (23).

Statistics

The data were asymmetrically distributed and analysed using the ordinal descriptive statistics. The results were expressed as median, interquartile ranges, and ranges (minimum-maximum). Statistical calculation was performed using the Microsoft® Office Excel. Mann-Whitney test was used to compare Der p 1 and Der f 1 levels (Statistica for Windows Release 5.5, StatSoft Inc.). Statistical significance was set up at $p<0.05$.

RESULTS

Levels of Der p 1 and Der f 1

Median allergen mass fractions in carpet samples were 0.32 μg g^{-1} [range (0.1-12.5) μg g^{-1}] for Der p 1 and 0.35 μg g^{-1} [range (0.1-31.2) μg g^{-1}] for Der f 1 (Table 1). Median value for the sum of allergen (Der p 1 + Der f 1) was 0.60 μg g^{-1}.

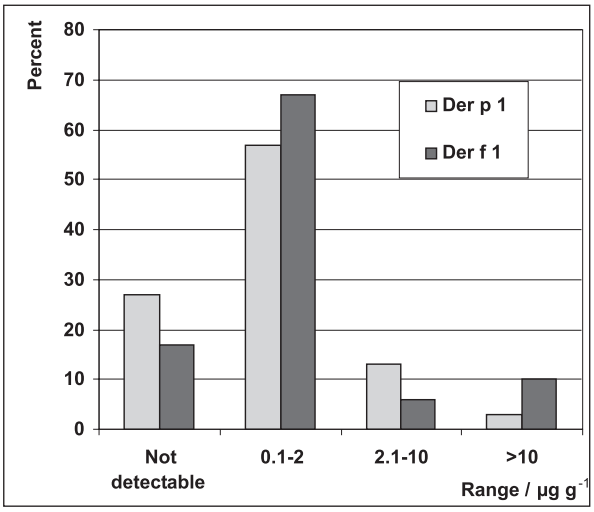

Figure 1 *Percentage (%) of homes (Zagreb) with Der p 1 and Der f 1 mass fraction ranges (μg g^{-1}) low 0.1-2, moderate 2.1-10, and high >10.*

Der p 1 and Der f 1 were below the limit of detection in 8 (27 %) and 5 (17 %) of homes, respectively (Figure 1). About 57 % and 67 % of homes contained Der p 1 and Der f 1 levels in the range of (0.1-2) μg g^{-1} (low level), respectively. Der p 1 at the lower cut point (>2 μg g^{-1}) were measured in four homes, whereas in one home the level of the allergen was above the upper cut point (>10 μg g^{-1}). Der f 1 in levels above the lower and upper cut points were found in two and three homes, respectively. The sum of allergen (Der p 1 + Der f 1) exceeded the lower threshold in eight samples (27 %). No statistical difference was found between the levels of Der p 1 and Der f 1 allergens in dust samples (Mann-Whitney test, $p>0.05$).

Table 1 *Median and ranges (μg g^{-1}) of Der p 1 and Der f 1 in carpet dust in 30 urban homes in Zagreb.*

Allergen	Mass fraction / μg g^{-1}		
	Median	Interquartile range	Range
Der p1	0.32	0.20-1.24	0.1-12.5
Der f 1	0.35	0.25-0.55	0.1-31.2
Der p 1 + Der f 1	0.60	0.37-2.11	0.1-31.2

Table 2 *Precision, accuracy, and limit of detection of the ELISA assay*

Allergen	CV (%)		Recovery / %	Detection limit / ng mL^{-1}
	intra-assay	inter-assay		
Der p 1	6.9	13.1	91	2
Der f 1	4.6	12	93	2

Analytical evaluation of ELISA assay

Table 2 shows the results of analytical evaluation of the ELISA method for determination of dust mite allergens. Intra-assay precision for 20 subsequent measurements was 4.6 % for Der p 1 and 6.9 % for Der f 1. Inter-assay precision for Der p 1 was 13.3 % and for Der f 1 12 %. The accuracy of Der p 1 and Der f 1 assays was from 91 % to 93 % (Table 2). The lower limit of detection for both allergens was 2 ng mL^{-1}. The range of linearity was (8-100) ng mL^{-1}.

DISCUSSION AND CONCLUSON

Based on Der p 1 and Der f 1 levels, the results were divided into four categories: below the limit of detection of the assay, low [(0.1-2) $\mu g\ g^{-1}$], moderate [(2.01-10) $\mu g\ g^{-1}$], and high level (>10 $\mu g\ g^{-1}$) (Figure 1) (24).

Our results showed that both mite allergens were present in the majority of the study homes in Zagreb (Figure 1). Only about 27 % and 17 % of homes had no detectable Der p 1 or Der f 1, respectively. The medians for Der p 1 and Der f 1 were 0.32 $\mu g\ g^{-1}$ and 0.35 $\mu g\ g^{-1}$, respectively (Table 1). About 57 % and 67 % of homes had Der p 1 and Der f 1 within the low range of (0.1-2) $\mu g\ g^{-1}$, respectively. These results show that Der p 1 and Der f 1 levels in carpet dust samples were generally low and are in accordance with our earlier study on urban Zagreb homes (Der p 1 and Der f 1 median <1 $\mu g\ g^{-1}$) which used the Dustscreen™ test (8). The ranges of Der p 1 and Der f 1 were (0.1-12.5) $\mu g\ g^{-1}$ and (0.1-31.2) $\mu g\ g^{-1}$, respectively, confirming similarly large ranges from earlier studies (8, 11-13, 20) for both allergens. In this study, dust mass fraction of Der p 1 and Der f 1 varied by an order of 10^2 between homes (Table 1). Additionally, the range of Der f 1 level was greater than that for Der p 1, as we expected (8). In urban homes with central heating and lower indoor relative humidity, *D. farinae* is the most prevalent mite due to its better physiological adaptation to dryer conditions (25).

Clinically significant Der p 1 or Der f 1 levels >2 $\mu g\ g^{-1}$ were found in the carpet dust of five (17 %) homes for each allergen (Figure 1). The sum of dust mite allergen (Der p 1 + Der f 1) exceeded this threshold in eight homes (27 %). Additionally, the sum of allergen (Der p 1 + Der f 1) was two times higher (median 0.60 $\mu g\ g^{-1}$) than single allergen measurement. Therefore, these results support other findings that determination of only one allergen provides limited information about mite infestation in homes in Zagreb and similar urban areas with moderate climate (2, 7, 8, 10).

Monoclonal antibody-based ELISA techniques have been established as the reference method for indoor allergen analysis. In this study, analytical evaluation of the ELISA assay included intra- and inter-assay precision, accuracy, and limit of detection. Precision analysis for Der p 1 and Der f 1 allergens shows that intra-assay CV for both allergens was in the range of 5 % to 7 %. Inter-assay variation for repeated assays was from 12 % to 13.1 % (Table 2). These results are consistent with the reported data by many authors (10, 13). The accuracy of both allergen measurements was from 91 % to 93 %, and these results are completely satisfactory (Table 1). In our experiments, the sensitivity of ELISA was increased by using the antibody pair of capture/detection monoclonal antibodies in the concentrations of 1.5 μg mL^{-1} and 1 μg mL^{-1}, respectively. The lower limit of detection was 2 ng mL^{-1}, which is consistent with other reports (26, 27). Therefore, our results have passed analytical validation for Der p 1 and Der f 1 concentrations in settled house dust.

Der p 1 and Der f 1 are the main allergens from mites *D. pteronyssinus* and *D. farinae* which are considered the most prevalent household mites all over the world (6-10). Accordingly, we choose them as the most convenient to measure mite allergen concentrations using the ELISA assay. Dust samples collected for exposure assessment of mite allergens should be stored at 4 °C for no longer than several days (21) or at -20 °C for up to 10 months before extraction and analysis (26).

As this study has not included dwelling conditions (humidity, ventilation, frequency of vacuuming, dampness, the age of the carpet, a household dog or cat) associated with high dust allergen levels, we need to stress that our results belong to the non-selected sample of homes.

In conclusion, our results show that the levels of mite allergens Der p 1 and Der f 1 in Zagreb households are highly variable, and that most homes had at least one allergen. Our data may provide the essential basis for future studies based on the ELISA assay for determining other indoor allergens (cockroach, mould) which may contribute to the total household and occupational allergen load in Croatia.

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Sažetak

ODREĐIVANJE ALERGENA GRINJA U KUĆNOJ PRAŠINI ENZIM-IMUNOKEMIJSKOM METODOM

Cilj ovoga rada bio je odrediti razine alergena grinja *Dermatophagoides pteronyssinus* (Der p 1) i *Dermatophagoides farinae* (Der f 1) u 30 uzoraka kućne prašine uzorkovane u gradskim stanovima u Zagrebu enzim-imunokemijskom metodom (ELISA, Indoor Biotechnologies Ltd, Carfiff, UK). Metoda se koristi tehnikom dvaju monoklonskih protutijela, a primjenjuje se kao standardna metoda za određivanje alergena grinja u prašini. Uzorci prašine skupljeni su usisavanjem prašine s tepiha u celulozne filtre. Maseni udjeli Der p 1 bili su u rasponu od 0.10 μg g^{-1} do 12.5 μg g^{-1} (medijan 0.32 μg g^{-1}), a Der f 1 od 0.10 μg g^{-1} do 31.2 μg g^{-1} (medijan 0,35 μg g^{-1}). Prisutnost Der p 1 i Der f 1 (>2 μg g^{-1}) u kućnoj prašini povezana je s povećanim rizikom od senzibilizacije alergenima grinja. U ovom radu je u oko 16 % stanova ustanovljena ta razina, i to za oba alergena. Ako se zbroje maseni udjeli alergena (Der p 1 + Der f 1), tada je razina alergena u kućnoj prašini >2 μg g^{-1} nađena u oko 27 % stanova.
Analitičkom procjenom metode ELISA dobiveni su zadovoljavajući rezultati za preciznost u seriji (KV < 6,9 %), preciznost iz dana u dan (KV < 13,3 %), točnost (91 % do 93 %) te osjetljivost (2 ng mL^{-1}). Metoda ELISA čini nužnu bazu za naša buduća ispitivanja alergena unutarnjih prostora.

KLJUČNE RIJEČI: *alergeni unutarnjih prostora, Der f 1, Der p 1, ELISA, grinje kućne prašine*

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