Original Article

DOI: 10.5582/bst.2013.v7.2.82

Detection of group 2 *Dermatophagoides pteronyssinus* allergen for environmental monitoring of dust mite infestation

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Summary **Aeroallergen avoidance has been promoted in order to prevent sensitization and the correlation between the level of allergen exposure and sensitization has been reported. The aims of this study were to monitor environmental mite infestation and to design an effective Der p 2 detection kit to estimate the number of mites in house dust samples. House dust samples were collected from 6 carpets and 2 mattresses monthly from April 2010 to March 2011. The total number of mites was counted under microscopes and Der p 2 concentrations were measured using Der p 2 ELISA kits. The detection kit was constituted using Der p 2 specific mouse monoclonal antibody as capture antibody, and rabbit polyclonal antibody as detection antibody. Both Der p crude extract and rDer p 2 were used as internal standards. The number of mites in the dust samples was significantly higher in the mattresses as compared with that in the carpets and the total number of dust mites was higher in the summer than any other seasons. The concentration of Der p 2 components in Der p crude extract was analyzed and the results showed that each gram of Der p crude extract contained 53.4 mg of Der p 2. When the number of mites and Der p 2 concentration were measured for the correlation analysis, the results showed that there was a good correlation between Der p 2 and number of mites with $R^2 = 0.9667$. Dust mites were significantly increased in the dust samples collected from mattresses especially in the summer. The good correlation between Der p 2 concentration and mite numbers indicated that the measurement of Der p 2 can be used to replace direct mite counting. Using the Der p 2 detection method to monitor environmental mite infestation may be beneficial for allergic subjects to prevent disease activation.**

Keywords: *Dermatophagoides pteronyssinus*, Der p 2, house dust mites, environmental allergen

1. Introduction

The prevalence of asthma in Taiwan has increased from 1.3% in 1974, to 13.1% in 2004 with a one percent increase annually over the past decade (*1*). The predominant dust mites in Taiwan that have been implicated in allergy are *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* (*2*). The climate is warm (average temperature: 22°C) and humid (average relative humidity: 76%), more than 90% of children with asthma have positive skin test responses to house dust mite (HDM), and *D. pteronyssinus* was found to be the primary species accounting for 78% of house dust mite (HDM) in Taipei (*3*).

At least 22 allergenic components in house dust mites have been cloned and identified (*4*). Among them, more than 80% of the HDM-allergic children with asthma have positive skin test reactions to group 1 and group 2 (Der p 1 and Der p 2) (*5*). Both *in vitro* IgE binding studies and *in vivo* skin tests revealed that more than 90% of the allergic children with asthma and mite allergy in Taiwan reacted to both Der p 1 and Der p 2 (*6*).

The occurrence and severity of asthma symptoms are related to environmental allergens (*7*). Aeroallergen

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avoidance has been promoted in order to avoid sensitization, and a correlation between the level of allergen exposure and sensitization has been shown previously (*8*). It is important to monitor the environmental allergen Der p in house dust. Our previous findings showed that dust mites increased in July and August and decreased in the winter, as determined by direct mite count in the dust sample collected from households of asthmatic patients (*2*).

It has been reported that a seasonal variation of Der p 1 was found as determined by ELISA based immunoassay. However, there were no seasonal variations in Der p 5 level (*5,6*). This discrepancy may be due to the different characteristics of Der p 1 and Der p 5. The protein concentration of each allergenic component might be different due to their enzymatic activity in the dust. Since it has been reported that Der p 2 was present in the mite body and can be used as an indicator of number of mites in the dust samples, it is feasible to use Der p 2 concentration to reflect mite count in the dust (*9*). A variety of techniques have been developed to measure levels of specific allergens in the environment using ELISA-based immunoassays for major allergens, such as Der p and Der f 1 and 2, Fel d 1, Bla g 1 and 2, Can f 1, Mus m 1, and Rat n 1 (*10*). There were high sequences of homology regarding mite group 2 allergens (Der p 2 and Der f 2) according to structure analysis. The crude extracts of *D. pteronyssinus* and *D. farinae* could be recognized by MoAb-C1 (generated by Der p 2) with a molecular weight of 16 kDa in our previous study, and therefore it is feasible to use these antibodies which are prepared from group 2 allergen to detect group 2 allergen in house dust (*11*). In this study both monoclonal antibody and polyclonal antibodies were generated for the development of the Der p 2 ELISA kit. The aim of this study was to develop an effective detection kit to measure Der p 2 concentrations in the dust samples and to correlate these concentrations with mite numbers counted using an inverted phase microscope.

2. Materials and Methods

2.1. *Preparation of mite extracts*

D. pteronyssinus was obtained from Allergen Pharmacia (Uppsala, Sweden). Mites were extracted by homogenizing after resuspending in phosphate-buffered saline (PBS) containing aprotinin (0.1 IU/mL; Sigma Chemicals, St. Louis, MO, USA) and phenylmethylsulfonyl fluoride (1 mmol/L; Sigma). After centrifugation at 10,000 g for 30 min, the supernatant was dialyzed against 0.05 mol/L of ammonium carbonate, pH 8.0. It was then freeze-dried in aliquots and stored at 4°C until use. The protein content was determined by Lowry's method, using bovine serum albumin as is standard.

2.2. *Recombinant Der p 2 preparation*

The cDNA of group 2 *D. pteronyssinus* allergen (Der p 2) was cloned into the commercial expression vector pIC9 (Invitrogen, USA) for the extracellular expression in the yeast *Pichia pastoris* according to the instruction manual. Expression of rDer p 2 by fermentation cultures was generated. In brief, fermentation was performed in a 1,000 mL flask with 200 mL buffered glycerol-complex (BMGY) medium (Invitrogen, USA). The temperature was maintained at 30°C and the concentration of dissolved oxygen (DO) was maintained at 35% by DO-agitation. The pH was adjusted to 7.0 by 15% NH_3H_2O. Fermentation was started by adding 200 μL of seed culture to the 200 mL of BMGY. After complete consumption of glycerol in the medium, a glycerol fed-batch phase was initiated by addition of 50% glycerol. Expression of recombinant Der p 2 was induced by the addition of methanol after glycerol was exhausted again. Purified recombinant protein Der p 2 was prepared as previous described (*12*). The cell-free supernatant was collected and the protein components were fractionated through ammonium sulfate (50%) precipitation and centrifugation. The pellet was dissolved in PBS buffer and dialysis against PBS buffer. The proteins will be then concentrated by Amicon Ultra centrifugal filter devices with an exclusion size of 10 kDa (Millipore, Bedford, MA). The addition rate was adjusted according to pH and DO. One milliliter of the culture was withdrawn to determine rDer p 2 expression using SDS-PAGE analysis.

2.3. *Monoclonal and polyclonal antibody production*

Monoclonal antibodies (MoAbs named as C1) against Der p 2 were prepared as previously described (*13*) and its immunoglobulin isotype belonged to IgG1 kappa. Briefly, spleen cells obtained from BALB/c mice immunized with the rDer p 2 were fused with murine plasmacytoma NS-1 cells in the presence of polyethylene glycol (molecular weight 1, 500 daltons; Merck, Hohenbrunn, Germany). Antibody-producing hybrid cells were screened using the enzyme-linked immunosorbent assay (ELISA) and recombinant GST-Der p 2 and GST alone. Briefly, hybridomas producing MoAbs were expanded and the Der p 2 specificity of the MoAbs was determined by ELISA with rDer p 2.

Polyclonal antibody anti-rDer p 2 was obtained from a New Zealand rabbit, which was injected once subcutaneously with rDer p 2 emulsified with primary complete Freund's adjuvant (10 mL, Sigma, St. Louis, MO, USA) and boosted incomplete Freund's adjuvant (10 mL, Sigma, St. Louis, MO, USA) once in every two weeks for a total of 3 times. Rabbit serum was obtained and store in −80°C before use, after the last immunization.

2.4. *SDS-PAGE and immunoblotting analysis*

In order to identify the components recognized by antibodies, protein components were separated and performed immunoblotting with MoAb (C1), polyclonal antibody and human IgE antibody. The *D. pteronyssinus* crude extract and rDer p 2 were separated by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane (Millipore, Chelmsford, MA). After being blocked with skimmed milk, membranes were reacted with anti-rDer p 2 MoAb (C1) from mice (1:1,000 dilution), polyclonal antibodies from rabbits (1:2,500 dilution), or serum samples from patients (1:5 dilution). Blots were then immersed with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (1:1,000 dilution) (Biosource, Camarillo, CA), goat anti-human IgE antibodies (1:1,000 dilution) (Biosource, Camarillo, CA) or goat anti-rabbit IgG antibodies (1:5,000 dilution) (Chemicon, Temecula, CA) and washed with PBS after each treatment. Finally, blots were immersed with enhanced chemiluminescence reagent (Pierce, Rockford, IL) for 2 min and exposed to X-ray films.

2.5. *Collection of dust and counting of mites from dust sample*

Random samples were collected every month between April 2010 and March 2011 from eight sample sites (six carpets from conference rooms and the library and two mattresses in the on-duty room) in a hospital environment. About 1 m^2 of surface area of each site was vacuumed for 1 min, following which each dust sample in the container of the vacuum cleaner was brushed completely into a plastic bag and analyzed. Zero point one gram from each sample was isolated and added to one milliliter of PBS. The suspension was poured through a 45-μm-pore filter and the material remaining on the filter, rinsed in an integrid petri-dish for mite number counting. Additional dust samples were picked up and extracted for the measurements of Dp and Der p 2 concentration by ELISA as described continuously. Mites were counted under an inverted phase contrast microscope (OLYMPUS SZ-PT, Japan) and the mite concentration was expressed as the number of mites per 1 g of dust. In order to properly identify and count the mites, the mites were picked up by hand using a 25-gauge needle under an incident light microscope, then immersed in polyvinyl alcohol solution on a microscope slide and covered with a glass cover.

2.6. *ELISA*

ELISA was performed as previously described (*13*). The C1 monoclonal antibody used were coated separately onto wells of polyvinyl microtiter plates (Costar, Cambridge, Mass., USA) by the addition of 100 μL of a 0.4 μg/mL solution of C1 monoclonal antibody in PBS, pH 8.0 for 3 h at RT. After blocking with 1% skimmed milk, it was then incubated for 1 h at RT. Wells were washed with PBS containing 0.05% Tween-20 (Southern Biotech Association, Birmingham, Ala., USA) (PBST). Zero point one gram of dust from each sample was added in 25 mL PBS, and the standard response curves were constructed from reference recombinant Der p 2 antigen solutions in the range of 62.5 ng/mL to 1,000 ng/mL. Wells were washed again with PBST, polyclonal antibody from rabbit serum (1:1,000 dilute in PBST) was added to the wells and incubated for 2 h at RT. After washing with PBST, peroxidase-conjugated goat anti-rabbit IgG were added to each well and were incubated for 1 h at 37°C, and the bound enzyme substrate were detected with ABTS substrate (Invitrogen, USA). The reaction was stopped with 50 μL 0.01% sodium azide (NaN_3) after 15 min, and the optical density were measured at 450 nm in a multiscan spectrophotometer (Sunrise, TECAN, Switzerland). Results were expressed as EU.

2.7. *Statistical analysis*

Results of mite numbers were expressed as a mean for each sampling locations. Data of seasonal variations at different distributions were represent as mean ± 2 × S.D. for each groups. The differences between groups were analyzed with a non-paired *t*-test. *p*-values less than 0.05 were considered statistically significant. Correlations were analyzed using Pearson's χ^2 test in the allergen protein concentrations and mite numbers.

3. Results

3.1. *Mite distribution in the house dust samples*

The number of mites in the house dust samples collected from two carpets and six mattresses were analyzed. A total of 12 specimens from April 2010 until March 2011 were collected in each sampling locations. The results showed that there were significantly more mites in the mattresses than in the carpets for the four seasons (Figures 1 and 2). Seasonal variation of mite count was also observed in the mattresses (Table 1). The mite population in the mattresses was higher in the summer as compared with other seasons (Figure 2).

3.2. *Protein profiles and Western blot analysis of D. pteronyssinus crude extracts*

The protein profiles of *D. pteronyssinus* crude extracts or rDer p 2 on SDS-PAGE showed several protein bands existing in the crude extracts and one single

protein existing in the recombinant protein (Figure 3A). In the crude extracts of *D. pteronyssinus*, a molecular weight of 16 kDa could be reacted with by rabbit anti-Der p 2 polyclonal antibodies and mouse anti-Der p 2 monoclonal antibodies. A strong signal either existed in rDer p 2 which reacting with rabbit anti-Der p 2 polyclonal antibodies and mouse anti-Der p 2 monoclonal antibodies (Figure 3B). Several IgE-binding proteins in the *D. pteronyssinus* crude extract as determined by the *D. pteronyssinus*-sensitive sera, only one major allergen with molecular weight around 16 kDa could be detected. Neither *D. pteronyssinu* crude extract nor rDer p 2 was observed in the serum from the healthy individual.

3.3. *Der p 2 concentration in D. pteronyssinus crude extract*

The mouse anti-Der p 2 monoclonal antibody-C1 used for capture antibody and the rabbit anti-Der p 2

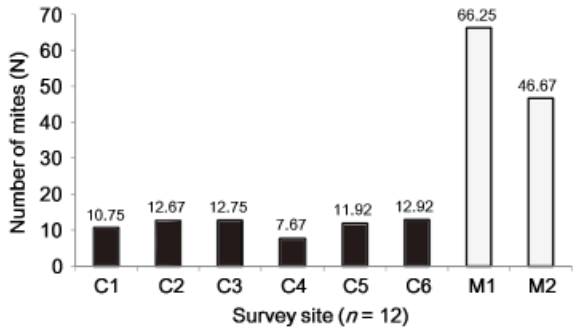

Figure 1. The number of mites from different survey sites included carpets (C1-C6) and mattresses (M1-M2). Mite numbers represent the samples taken from the carpets (black bars) and represent samples taken on the mattresses (white bars). A total of 12 specimens from April 2010 until March 2011 were collected in each sampling locations.

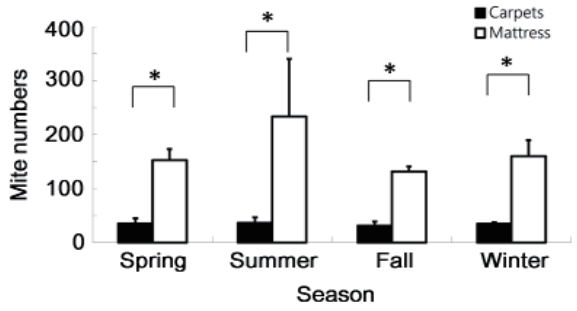

Figure 2. Seasonal variation of the distribution of mite number at different indoors locations. Mite numbers represent the samples taken from the carpets (black bars) and represent samples taken on the mattress (white bars). Data represent means ± S.D. in a single experiment, representative of an experimental *n* of 6. *$p < 0.05$.

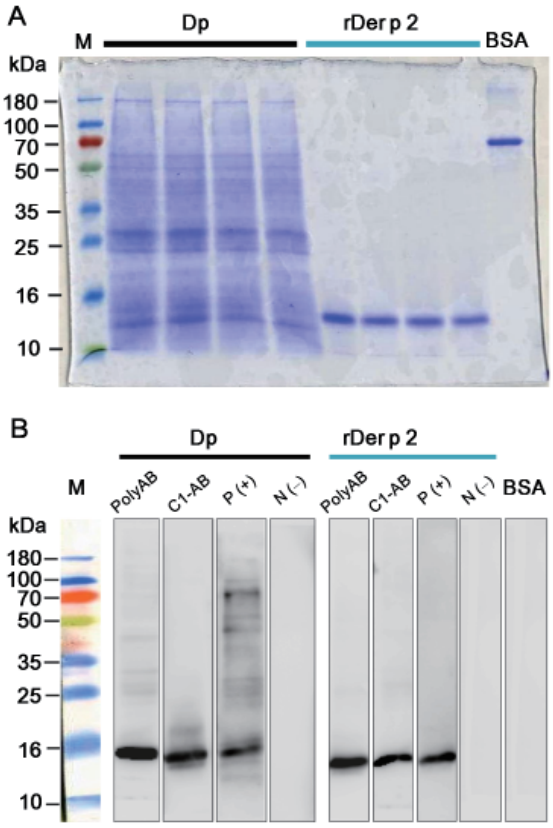

Figure 3. Protein profiles and Western blot of *D. pteronyssinus* crude extracts and recombinant Der p 2. (A), Proteins were analyzed on 12% SDS-PAGE and present with Coomassie blue staining. M: protein marker; Dp: *D. pteronyssinus* crude extracts; rDer p 2: recombinant Der p 2; BSA: Bovine serum albumin. **(B)**, Western blot analysis with different antibodies. PolyAB: rabbit anti-Der p 2 polyclonal Ab; C1-AB: mouse anti-Der p 2 monoclonal Ab; P(+): patient allergic to *D. pteronyssinus*; N(-): healthy individual.

Table 1. Number of mites isolated from dust samples collected from two mattresses (M1, M2) and six carpets (C1–C6) from April 2010 until March 2011

Sample	2010									2011		
No.	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
C1	160[#]	80	130	90	110	150	90	60	110	130	110	70
C2	80	190	120	150	190	80	70	110	170	90	130	140
C3	180	60	90	40	260	190	140	80	130	120	80	160
C4	80	20	40	80	60	40	110	100	90	150	70	80
C5	110	160	180	160	100	70	100	90	60	170	110	120
C6	90	160	140	80	140	120	90	190	150	80	130	180
M1	600	200[*]	540	670	370	320	250	680	550	490	350	580
M2	640	480	1,580	1,030	480	420	370	590	690	630	490	550

[#] Number of mites/1 g; C: Carpet; M: Mattress; [*]The dust sample was collected after the mattress and the duty room was cleaned.

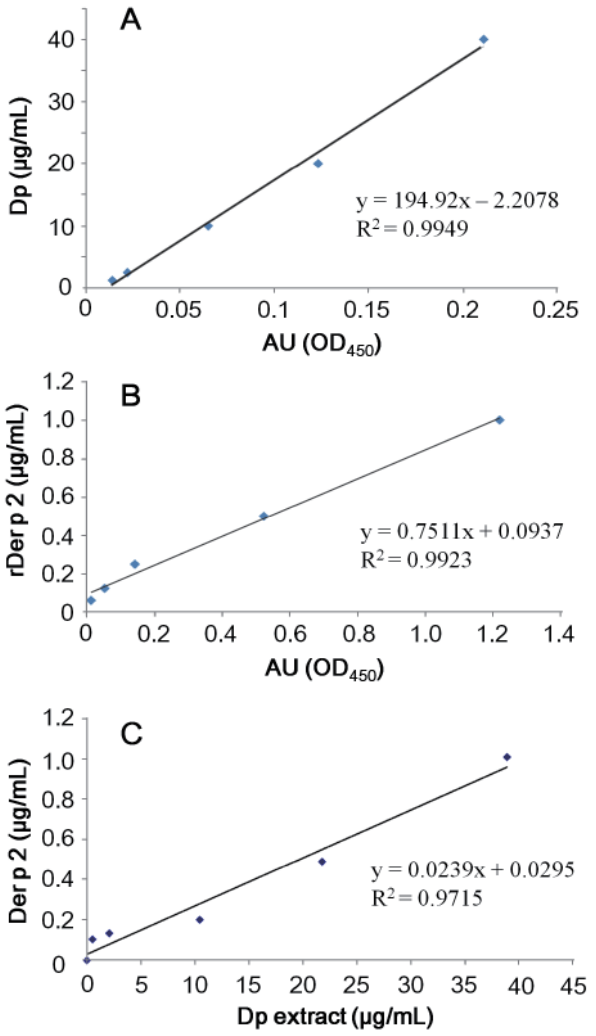

Figure 4. Der p 2 protein concentrations in the extract of *D. pteronyssinus*. Der p 2 mouse monoclonal antibody C1 was used for capture antibody, rabbit polyclonal antibody was used for detection antibody and rDer p 2 was used as protein standard. The correlation between *D. pteronyssinus* protein concentration and ELISA Absorbance Units (AU) OD450 nm was determined **(A)**, the correlation between Der p 2 concentration and AU(OD450 nm) was determined **(B)**, and the correlation between Der p 2 and *D. pteronyssinus* extract was determined **(C)**.

polyclonal antibody used for detection antibody were performed to detect the concentrations of Der p 2 in the environmental samples. The known concentrations of *D. pteronyssinus* crude extract or rDer p 2 were used as protein standard. The known protein concentrations of *D. pteronyssinus* crude extract or rDer p 2 were measured by ELISA analysis, and the results of ELISA data were presented in absorption unit (AU) OD450 nm as standard curve (Figures 4A and 4B). The correlation between known *D. pteronyssinus* protein concentration and ELISA absorbance units (AU) OD450 nm was determined (Figure 4A), the correlation between known rDer p 2 concentration and ELISA AU OD450 nm was determined (Figure 4B), and the correlation between Der p 2 and *D. pteronyssinus* extract was determined (Figure 4C).

When series dilution of *D. pteronyssinus* crude extracts were measured for the Der p 2 protein concentrations, the results showed that each gram of *D. pteronyssinus* crude extract contains 53.4 mg of Der p 2 (Figure 4C). For the development of Der p 2 ELISA kit, there were good correlation between rDer p 2 protein concentrations with OD 450 nm ELISA unit (R^2 = 0.9923) and the detectable range of rDer p 2 was between 62.5 ng/mL and 1 µg/mL (Figure 4B).

3.4. *The correlation of mite number with Der p 2 protein concentration in house dust samples*

A total of 12 house dust samples were selected for the correlation analysis. The Dp or Der p 2 concentrations were determined using ELISA with anti-Der p 2 MoAbs C1. The data were calculated by the formula from Figures 4A and 4C as showed in the Table 2. The Dp or Der p 2 protein concentrations in the house dust samples were measured after the mite numbers were determined. The results showed that there was good correlation (R^2 = 0.9667) between Der p 2 protein concentrations and mite numbers (Figure 5).

Table 2. Measurements of allergen concentrations and mite numbers in the dust samples

Year/Month	Sample No.	Dp (µg/mL)	Der p 2 (µg/mL)	Dp (µg/g of dust)	Der p (2 µg/g of dust)	Number of Mites (*n*)
2010 Jun	M1	15.92[a]	0.42[b]	159.22[c]	4.21[d]	54[e]
	M2	36.19	0.89	361.91	8.93	158
2010 Jul	M1	21.96	0.56	219.63	5.64	67
	M2	26.06	0.65	260.61	6.52	103
2010 May	M1	9.86	0.27	98.60	2.71	20
	M2	15.19	0.40	151.91	4.02	48
2011 Jan	C1	5.59	0.17	55.91	1.72	13
	M1	14.36	0.38	143.60	3.80	49
	M2	19.62	0.50	196.23	5.01	63
2011 Feb	C1	5.39	0.16	53.94	1.58	13
	M1	12.21	0.32	122.16	3.21	35
	M2	14.56	0.37	145.56	3.78	49

[a]: Data were calculated by the formula "y = 194.92 x - 2.2078" from the Figure 4A; [b]: Data were calculated by the formula "y = 0.0239 x + 0.0295" from the Figure 4C; [c]: 1 g dust/10 mL PBS; [d]: 1 g dust/ 10 mL PBS; [e]: The correlation between Der p 2 protein concentrations and mite numbers was performed to acquire the formula "y = 201.47 x - 31.412" as showed in the Figure 5.

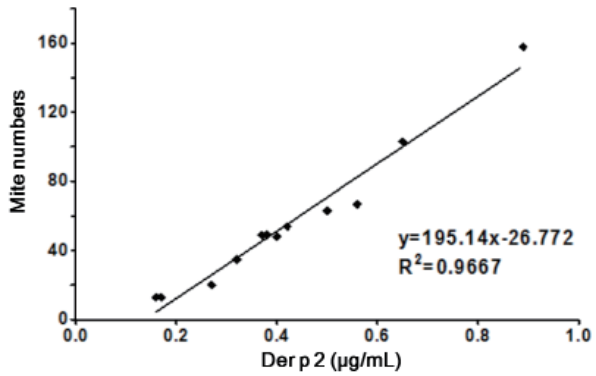

Figure 5. The correlation of Der p 2 protein concentrations and mite number were analyzed. A total of twelve dust samples were selected for the correlation analyzes Der p 2 ELISA kit was used to measure the protein concentration of Der p 2 (μg/mL). Number of mite was counted under inverted phase contrast microscope. The correlation were obtained by linear fit analysis $R^2 = 0.9667$ ($p < 0.001$).

4. Discussion

In this study, we demonstrated that house dust mites could be identified in a hospital environment, particularly in the mattresses of the on-duty room, which had a significantly higher number of mites as compared with those in the carpets from the conference rooms and the library. A similar finding was observed in our previous report that the number of mites in the mattresses in the bedroom was more prominent than in the carpets of the sitting room (*2*). Both species of dust mite *D. pteronyssinus* and *D. farinae* were identified in the house dust as our previously described (data not shown) (*2*). Although the number of mites increased in summer, there were no significant changes as compared to other seasons, and this discrepancy may be due to the constant room temperature maintained by air-conditioning in the on-duty room in the summer.

The composition analysis of Der p 2 in the *D. pteronyssinus* crude extract showed that the concentration of Der p 2 in whole mite extracts was 53.4 mg/g, indicating that there was only a trivial amount of Der p 2 in the whole body extract. Despite the trivial amount of Der p 2, more than 80% Der p sensitive asthmatics have Der p 2 specific IgE antibodies. This result indicated that Der p 2 may play an important role in *D. pteronyssinus* sensitization. In previous study, we have demonstrated that Tyr p 2 is the major allergenic component in storage mite *Tyrophagus putrescentiae* of allergic rhinitis patients and processes high level cross-reactivity with Der p 2 (*14*). The IgE-binding titers of group 2 allergens were well correlated and the binding activity of Tyr p 2 could be absorbed by Der p 2 (*14*). Indeed, group 2 allergens are more cross-reactive not only between dust mites *D. pteronyssinus* and *D. farinae*, but also with other storage mites *T. putrescentiae* and *Lepidoglyphus destructor* (*15*). However, there was few storage mites been found in dust samples collected from the hospital carpets and mattress in this study. Similar results had been reported that nearly bedding samples collected in Korea homes were found to contain a large number of house dust mite but few of storage mite *T. putrescentiae* (*16*). House dust mites *D. pteronyssinus* and *D. farina* are the major species in the bedding and bedroom floor, however, storage mite *T. putrescentiae* mainly identified in the kitchen floor. Although the group 2 mite allergens with a high level of cross-reactivity, it does not interfere the results from different sampling locations.

Previous studies have demonstrated that Der p 1 and Der p 5 can be detected in the house dust samples (*5,6*). However, these reports did not show the correlations between the concentration of Der p 1 and Der p 5 with the actual number of house dust mites in the environment (*5,6*). Our study demonstrated that Der p 2 concentrations had a strong correlation with the number of mites in the house dust samples. This result indicated that Der p 2 might be more relevant to mite infestation in the environment. Although the non-enzymatic characteristics of Der p 2 might be different from the enzymatic allergens of house dust mites, it cannot be clarified unless the two types of allergens in the dust samples are measured simultaneously. Since Der p 1 was not analyzed in this study, whether it can be used to reflect the number of mites remains unclear and requires further investigation.

The developments of allergic diseases are directly related to allergen levels, such as mite density in house dust. The best way of dealing with allergic disease is believed to be a combination of allergen diagnosis, environment control and medication. In this study, the strong correlation between Der p 2 concentrations and number of mites had been found indicated that the measurement of Der p 2 can be used to reflect mite count in the environment. The easy detection of mite number through Der p 2 measurement is an effective method of indoor allergen management. Allergen avoidance in habitant can reduce prevalence of allergic diseases (*16*).

Both monoclonal antibody and polyclonal antibodies were generated in this study for the development of the Der p 2 ELISA kit. The aim of this study was to develop an effective detection kit to measure Der p 2 concentrations in the dust samples and to correlate these concentrations with mite numbers counted using an inverted phase microscope. We developed an effective detection kit to measure Der p 2 concentrations in dust samples. The Der p 2 measurements of monitor mite infestation may be beneficial for allergic subjects to help to prevent disease activation. A systematic review indicates that extensive allergen control in bedrooms may reduce perennial allergic rhinitis symptoms induced by house-dust mite exposure (*17*). Therefore, the monitoring of dust mite infestation in the environment is important for asthmatic patients. In this study, a Der p

2 ELISA kit was developed and could accurately reflect the environment infestation of Der p, indicating that this Der p 2 ELISA kit could be used in a clinical setting.

Acknowledgements

The authors sincerely appreciate the assistance of the Biostatistics Task Force of Taichung Veterans General Hospital with regards to statistical assistance. This study was supported by grants (TCVGH-T997807) from the Taichung Veterans General Hospital and (VGHUST-99-G6-4) from the Veterans General Hospitals University System of Taiwan Joint Research Program.

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(*Received July 10, 2012; Revised March 4, 2013; Accepted April 7, 2013*)