

Establishment of Two Novel ELISA Methods for *Dermatophagoides farinae*-Specific IgE Detection with Recombinant Group 2 Allergen

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Abstract. *Dermatophagoides farinae*, or the American house dust mite, is a common cause of allergy and asthma. Current tests for sensitization to *D. farinae* include an indirect enzyme-linked immunosorbent assay (ELISA) method for specific IgE detection, which, while clinically useful, is time-consuming and has low sensitivity since it uses crude mite extracts. We developed two new ELISA methods to detect the group 2 allergen from *D. farinae* (Der f 2) and the Der f 2-specific IgE in sera of patients with asthma. Using recombinant Der f 2 protein for the analysis of Der f 2-specific IgE, we tested both indirect ELISA and avidin biotin complex ELISA (ABC-ELISA) methods in 46 patients who were also tested by Pharmacia UniCap. Both of these approaches are more specific than traditional methods using crude mite extracts. These new tests could aid in the laboratory diagnosis of asthma due to sensitization to *D. farinae*.

Introduction

Dermatophagoides commonly live in human habitations. Their excreta, metabolites, and bodies have strong allergenicities, causing allergic asthma, atopic dermatitis, allergic rhinitis, and other allergic diseases. Indeed, 10-20% of the global population suffers from these diseases [1, 2].

At least 23 groups of dust mite allergens have been identified in the two predominant dust mite species, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* (IUIS Allergen Nomenclature Sub-Committee <http://www.allergen.org/>). Allergen groups 1 and 2 are confirmed to be major components, binding to 80 to 90% of subjects who have antibodies to house dust mite extracts [3]. A variety of clinical examinations exist to detect sensitization to these allergens. *In vivo* allergen tests include the intradermal test, prick test [4], patch test, and provocation test [5]; *in vitro* experiments include the basophilic granulocyte histamine release experiment, radiation allergen absorption assay [6], Pharmacia Unicap [7], and ELISA [8].

ELISA is commonly used to analyze specific IgE in the patients' sera. The current application of indirect ELISA to determine Der f-specific IgE has been widely employed in clinical practice and scientific research. Although current methods are simple and require no special equipment, they require considerable time and have low sensitivity and specificity due to natural allergen extracts composed of complex components [9]. Thus, a new approach using recombinant allergens was tested to determine whether it would confer higher specificity and sensitivity and thus aid in the clinical diagnosis of dust mite allergies and asthma. Here, we describe two novel ELISA methods based on the development of recombinant *D. farinae* group 2 allergen.

Materials and Methods

Sample collection. Fasting venous blood (5 mL) was collected from 46 asthma patients diagnosed by the First Hospital of Shanghai, Shanghai Jiaotong University. The study Han Chinese population included 30 males and 16 females, ages 7 to 56 years (mean age 29.6±12.1 years), who had never received specific immunotherapy for *D. farinae*. All patients were tested with Pharmacia UniCap

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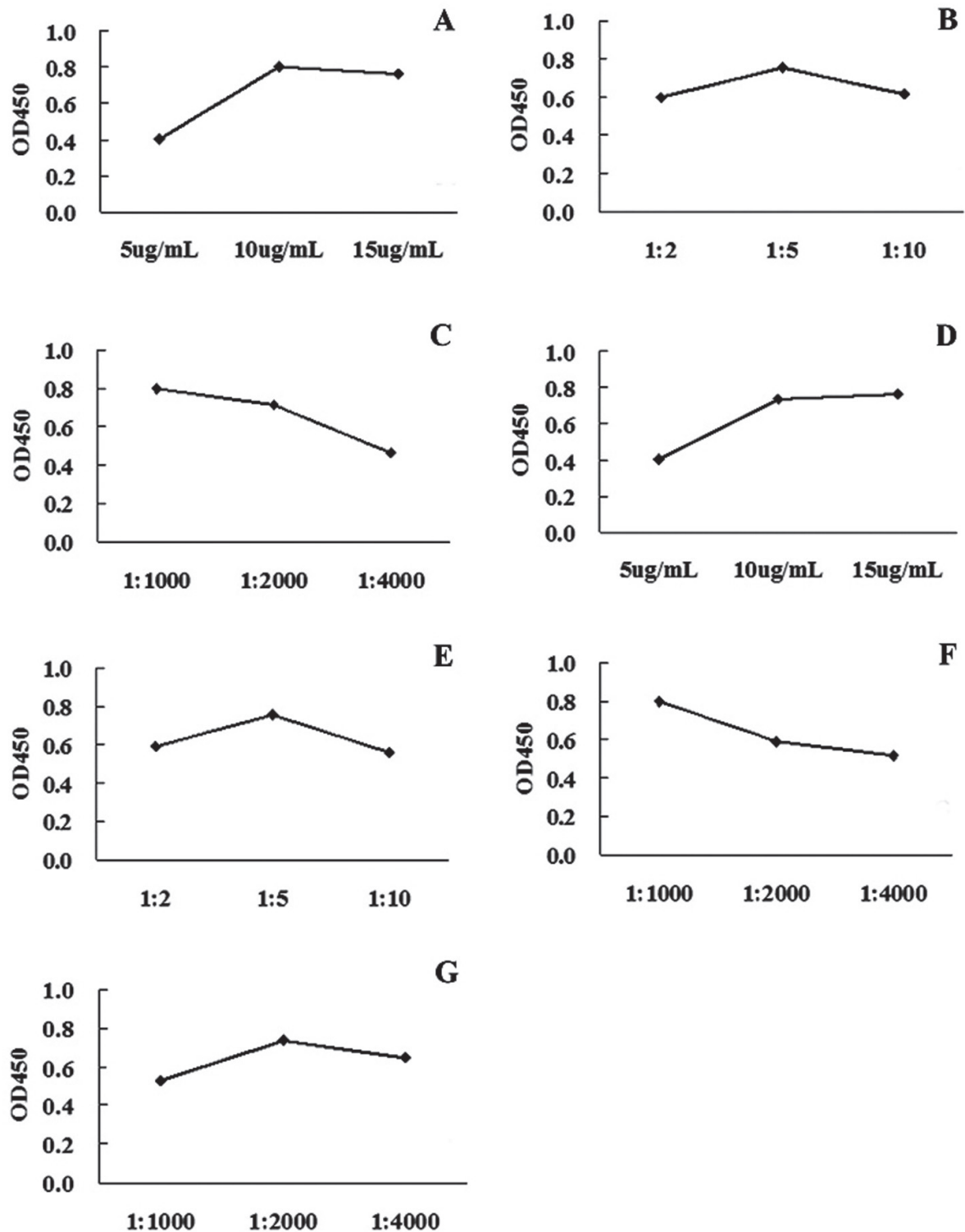


Figure 1. OD450 values for different test conditions (concentration or dilution) in indirect ELISA (A-C) and ABC ELISA (D-G) methods. A: Coating buffer; B: Serum; C: Anti-human IgE-HRP; D: Coating buffer; E: Serum; F: Anti-human IgE-biotin; G: streptavidin-HRP.

Table 1. Detection of Der f 2-specific IgE in sera of patients with asthma by indirect ELISA and ABC ELISA with recombinant Der f 2 protein.

UniCap Status	ABC ELISA	Indirect ELISA		Total
		+	-	
UniCap Pos	+	35	2	37
	-	1	1	2
UniCap Neg	+	0	0	0
	-	0	7	7
Total		36	10	46

for allergy to *D. farinae*; 39 patients tested positive (UniCap Pos) and 7 tested negative (UniCap Neg) (see [10] for determination criteria). Serum was separated from each sample, then stored at -80 °C for future use. Positive reference serum was a pool of 5 serum samples from children with asthma who had positive skin tests to recombinant Der f 2 and had never received specific immunotherapy. Negative reference serum was a pool of 5 umbilical serum samples from children whose mothers had no genetic allergic history. Human subjects research was approved by the Institutional Review Board of the First Hospital of Shanghai, Shanghai Jiaotong University.

Recombinant protein. Recombinant *D. farinae* group 2 (Der f 2) allergen protein was developed according to previous methods [11]. Recombinant Der f 2 was diluted to working concentrations of 5, 10, and 15 µg/mL.

Indirect ELISA. For indirect ELISA, 100 µL of Der f 2 recombinant protein were added in 48-well microtiter plates as coating buffer. Plates were placed at 4 °C overnight, then washed 3 times for 5 minutes each with washing liquid (1× PBS solution, 0.02% Tween-20). Next, wells were covered with 300 µL sealing solution (wash buffer containing 3% BSA) and incubated at 4 °C overnight. 100 µL reference sera was added before incubation at 37 °C for 2 hours, followed by washes as described above. For detection, 100 µL anti-human IgE-HRP (Sigma, St. Louis, MO) was added. Plates were then incubated at 37 °C for 2 hours and washed as previously described. 100 µL enzyme

substrate solution (tramethylbenzidine, TMB) was added to develop the HRP, and the plates were incubated at 37 °C for 30 minutes. 100 µL stop solution (2NH₂SO₄) was added. ELISA analyzer (RT-2100C, Lorderan Scientific Instrument Co., Ltd., Shanghai, China) was zeroed with PBS. Optical density was measured at 450 nm wavelength (OD₄₅₀) for 3 wells. The 3-factor and 3-level orthogonal table was used to design the crossover trial protocol with various factors. The 3 factors (three levels) were coating buffer concentration (5, 10, 15 µg/mL), serum dilutions (1:1, 1:5, 1:10), and anti-human IgE-HRP (1:1000, 1:2000, 1:4000).

ABC ELISA. For ABC ELISA, conditions were identical except that instead of anti-human IgE-HRP, 100 µL anti-human IgE-Biotin (Sigma) was added, after which plates were incubated at 37°C for 2 hours and washed as outlined above; then 100µL of Streptavidin-HRP (Sigma) was added, after which plates were again incubated at 37°C for 2 hours. Additionally, the 4-factor and 3-level orthogonal table was used to design the crossover trial protocol with various factors. The 4 factors (three levels) were coating buffer concentration (5, 10, 15 µg/mL), serum dilutions (1:1, 1:5, 1:10), anti-human IgE-Biotin (1:1000, 1:2000, 1:4000), and Streptavidin-HRP (1:1000, 1:2000, 1:4000).

Statistics. Using SPSS13.0 statistical software, ANOVA analysis of the orthogonal design was used to screen optimal experimental conditions, and regression analysis was performed for positive standard serum results of Der f 2-specific IgE (two-sided tests, with test level $\alpha = 0.05$, and $P < 0.05$

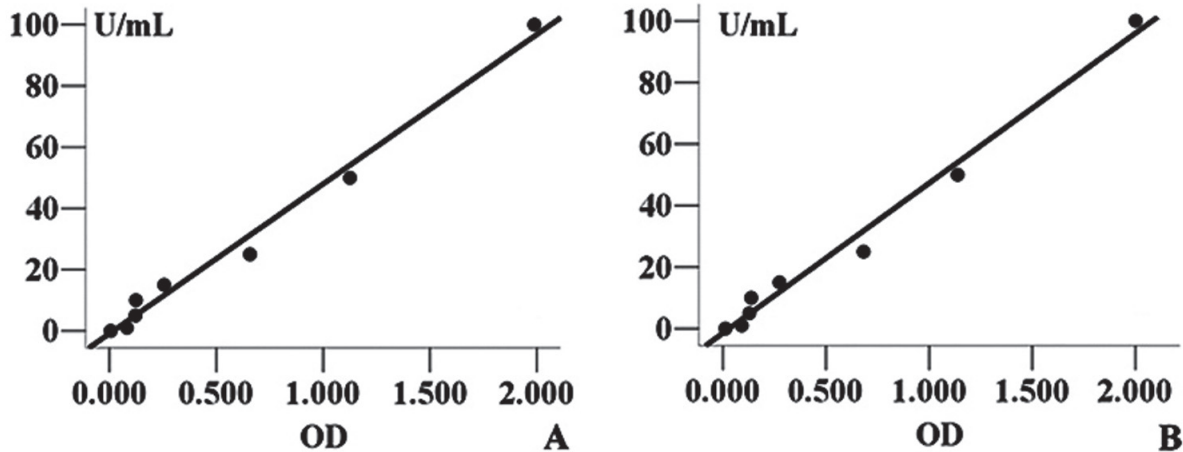


Figure 2. Standard curve of Der f 2-specific IgE. A: Indirect ELISA; B: ABC ELISA.

considered as statistically significant). Optimal ELISA methods were used to detect Der f 2-specific IgE positive standard serum and Der f 2-specific IgE in serum of patients with asthma. Each plate included positive, negative, reference serum, and PBS blank control, with 3 wells for each sample or reference serum, classified as positive and negative according to the international sIgE grading criteria [7].

Results

Optimal ELISA conditions. In considering the primary efficacy of indirect ELISA, coating buffer concentration ($F=107.490$), serum dilution ($F=16.247$), and anti-human IgE-HRP dilution ($F=66.725$) all had statistically significant effects on the experimental results ($P<0.05$). Optimal experimental conditions for this technique were as follows: coating buffer concentration of 10 $\mu\text{g/mL}$, serum dilution of 1:5, and anti-human IgE-HRP dilution of 1:1000 (**Figures 1A-C**). For ABC ELISA, coating buffer concentration ($F=2768.575$), serum dilution ($F=773.288$), anti-human IgE-Biotin dilution ($F=1443.772$), and streptavidin-HRP dilution ($F=731.209$) all had statistically significant effects on the experimental results ($P<0.05$). Optimal experimental conditions for ABC ELISA were as follows: coating buffer concentration of 15 $\mu\text{g/mL}$, serum dilution of 1:5, anti-human IgE-Biotin dilution of 1:1000, and streptavidin-HRP dilution of 1:2000 (**Figures 1D-G**).

Linear range and sensitivity. We also determined the linear range and sensitivity of indirect ELISA and ABC ELISA with recombinant Der f 2. Standard curves were made using standard serum (1.66U/mL–100U/mL) of Der f 2-specific IgE (Plasma Lab International; **Figure 2**). Regression analysis was performed for the measured OD values and Der f 2-specific IgE concentrations. Standard liquid (0 U/mL) was tested 10 times according to the ELISA sensitivity calculation method [12] to obtain mean optical density ($\bar{x}$) and standard deviation (s) at zero; $\bar{x} + 2s$ values were calculated by interpolation. The regression coefficients of indirect ELISA and ABC ELISA were 0.987 and 0.986, respectively, and the calculated sensitivity values were 0.72 U/mL and 0.69 U/mL.

Validation in human serum samples. Finally, we evaluated our test method using samples from 46 asthma patients (39 UniCap- positive and 7 UniCap-negative patients). Of 39 UniCap-positive patients, indirect ELISA confirmed 36 (92.3%) and ABC ELISA confirmed 37 (94.9%) with positive Der f 2-specific IgE in serum (**Table 1**). All 7 (100%) UniCap-negative patients tested negative for Der f 2-specific IgE in serum by both ELISA techniques. Coincidence rates of indirect ELISA and ABC ELISA methods were 93.5% (43/46 cases) and 95.7% (44/46 cases), respectively.

Discussion

Diagnosis of Der f-sensitized asthma relies on both *in vivo* and *in vitro* methods. *In vitro* application of allergen-specific IgE detection reagents, like the Pharmacia UniCap specific IgE allergen detection system, uses solid phase of polymer compounds combined with a large number of allergens. This approach has high sensitivity and good detection efficiency compared to *in vivo* methods; however, the disadvantage of this method is that allergen protein cannot be standardized nor made highly specific.

Here, we designed the Der f 2-specific IgE detection reagents based on classical ELISA principles, which can quantitatively analyze Der f 2-specific IgE in serum with higher sensitivity and specificity than traditional clinical tests using crude mite extracts. The ELISA microporous coating has sufficient amounts of purified allergen proteins, thus, during niche targeting determination of one specific allergen IgE in patient serum, ELISA has sensitivity comparable to that of Pharmacia UniCap. Purified recombinant and expressed allergen proteins, like Der f 2, can solve the standardization problem, representing a major improvement over other systems, including Pharmacia UniCap.

Our indirect ELISA and ABC ELISA methods, using this recombinant protein as a coating agent, produced sensitivities of 0.72 U/mL and 0.69 U/mL and coincidence rates of 93.5% and 95.7%, respectively. Thus, the established ELISA methods using recombinant protein have good sensitivity and coincidence rates in detecting Der f 2-specific IgE, offering a diagnostic basis for asthma induced by the Der f allergen. Additionally, if the HRP-labeled anti-IgG antibody is used instead of the HRP-labeled anti-IgE antibody, this method can detect concentrations of specific sub-type IgG generated by the body during mite-specific immunotherapy. Even though allergen protein microarray technology is now being used to screen for allergen sensitivity in patients [13], the allergen-specific IgE ELISA methods established in this study may still improve diagnosis of the disease source. In addition, these methods can further be applied to detect the therapeutic outcomes of specific immunotherapy.

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References

1. Derewenda U, Li J, Derewenda Z, Dauter Z, Mueller GA, Rule GS, and Benjamin D, The crystal structure of a major dust allergen Der p 2, and its biological implications. *J Mol Biol*, 2002. 318(2):p.189-97
2. Platts-Mills TA, Rakes G, and Heymann PW, The relevance of allergen exposure to the development of asthma in childhood. *J Allergy Clin Immunol*, 2000. 105(2Pt2):s.503-8.
3. Smith AM, Benjamin DC, Hozic N, Derewenda U, Smith WA, Thomas WR, Gafvelin G, van Hage-Hamsten M, and Chapman MD, The molecular basis of antigenic cross-reactivity between the group 2 mite allergens. *J Allergy Clin Immunol*, 2001, 107(6): p. 977-84
4. Karakaya G and Kalyoncu AF, The natural course of atopy determined by skin prick tests in patients with bronchial asthma and/or rhinitis. *Allergol Immunopathol (Madr)*, 2006. 34(6): p. 257-62.
5. Kanthawatana S, Maturim W, Fooanan S, and Trakultivakorn M, Skin prick reaction and nasal provocation response in diagnosis of nasal allergy to the house dust mite. *Ann Allergy Asthma Immunol*, 1997. 79(5): p. 427-30.
6. Price JA, Reiser J, Longbottom JL, and Warner JO, Inhalant allergy in asthmatic children: skin prick test, radioallergen sorbent test and chemiluminescent assay compared with allergen levels in their mattress dusts. *Int Arch Allergy Appl Immunol*, 1989. 88(1-2): p. 183-4.
7. Ricci G, Capelli M, Miniero R, Menna G, Zannarini L, Dillon P, and Masi M, A comparison of different allergometric tests, skin prick test, Pharmacia UniCAP and ADVIA Centaur, for diagnosis of allergic diseases in children. *Allergy*, 2003. 58(1): p. 38-45.
8. Peng Z, Xu W, and Simons FE, Highly sensitive and specific ELISA with monoclonal antibody capture to measure *Dermatophagoides farinae* 1-specific IgE. *Ann Allergy Asthma Immunol*, 1998. 80(3): p. 274-8.
9. Zhang C, Gjesing B, Spangfort MD, Xu J, and Zhong N, The allergen-specific IgE reactivity pattern of Chinese house dust mite allergic patients. *Allergy*, 2008. 63(12): p. 1640-1.
10. Silva DA, Gervasio AM, Sopelete MC, Arruda-Chaves E, Arruda LK, Chapman MD, Sung SS, and Taketomi EA, A sensitive reverse ELISA for the measurement of specific IgE to Der p 2, a major *Dermatophagoides pteronyssinus* allergen. *Ann Allergy Asthma Immunol*, 2001. 86(5): p. 545-50.
11. Yu-bao C, Zhou Y, Weihong S, Guifang M, Yang L, and Yungang W, Cloning, expression, and analysis of the group 2 allergen from *Dermatophagoides farinae* from China. *An Acad Bras Cienc*, 2010. 82(4): p. 941-51.
12. Grodzinsky E, Ivarsson A, Juto P, Olcen P, Falth-Magnusson K, Persson LA, and Hernell O, New automated immunoassay measuring immunoglobulin A antigliadin antibodies for prediction of celiac disease in childhood. *Clin Diagn Lab Immunol*, 2001. 8(3): p. 564-70.
13. Kim TE, Park SW, Cho NY, Choi SY, Yong TS, Nahm BH, Lee S, and Noh G, Quantitative measurement of serum allergen-specific IgE on protein chip. *Exp Mol Med*, 2002. 34(2): p. 152-8.