Enhanced sensitivity of capture IgE-ELISA based on a recombinant Der f 1/2 fusion protein for the detection of IgE antibodies targeting house dust mite allergens

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Abstract. The detection of allergen-specific immunoglobulin (IgE) is an important method for the diagnosis of IgE-mediated allergic diseases. The sensitivity of the indirect IgE-ELISA method against allergen extracts is limited by interference from high IgG titers and low quantities of effectual allergen components in extracts. To overcome these limitations, a novel capture IgE-ELISA based on a recombinant Der f 1/Der f 2 fusion protein (rDer f 1/2) was developed to enhance the sensitivity to IgEs that bind allergens from the house dust mite (HDM) species Dermatophagoides farinae. pET28-Der f 1/2 was expressed in the form of inclusion bodies comprising refolded protein, which were then purified. It exhibited increased IgE-specific binding (24/28, 85.8%) than rDer f 1 (21/28, 75.0%) or rDer f 2 (22/28, 78.6%) with HDM-allergic sera. Furthermore, in a random sample of HDM-allergic sera (n=71), capture-ELISA (71/71, 100%) was more sensitive than indirect-ELISA (68/71, 95.8%) for the detection of HDM-specific IgEs (P<0.01), indicating that this novel method may be useful for the diagnosis of HDM allergy.

Introduction

Allergic diseases, including allergic asthma, allergic rhinitis and allergic dermatitis, affect 30-40% of the world population; furthermore, incidence and mortality rates associated with allergic diseases are increasing, particularly within young populations (1). At present, >50% of allergic diseases are induced by house dust mites (HDMs), a common source of inhaled allergens (2). A total of 37 HDM allergen groups have been denominated (http://www.allergen.org); of these, the allergen groups 1 and 2 are the most clinically relevant, with >80 and >90% of patients with HDM allergies exhibiting an immunoglobulin (IgE) response to groups 1 and 2 HDM allergens, respectively (3).

IgE is an important pathogenic mediator of allergic immune responses. The detection of allergen-specific IgE is an effective diagnostic method and anti-IgE therapy is used to treat IgE-mediated allergic diseases (4). The indirect IgE-ELISA method is frequently used to detect allergen-specific IgE in serum samples, due to its simplicity and low cost compared with automated ImmunoCAP® systems; however, there are certain limitations (5). The normal range of the human serum levels of IgE is 50-300 ng/ml, which is notably low compared with that of IgG (~10 mg/ml) (6). In addition, the levels of HDM allergen-specific IgE, even in sera from patients with HDM allergies, are markedly decreased compared with the normal range of total IgE (7). Thus, the sensitivity of the indirect ELISA method is reduced by high titers of IgG that compete to bind with coated antigens (8). Furthermore, indirect ELISA frequently uses an HDM extract mixture as a coated antigen; the sensitivity of this method is reduced by the low amounts of effective allergen components in these mixtures. 

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Abbreviations: Der f 1, Group 1 allergen of Dermatophagoides farinae; Der f 2, Group 2 allergen of Dermatophagoides farinae; rDer f 1/2, recombinant Der f 1 and Der f 2 fusion protein; DSM, Difco skim milk; HDM, house dust mite; HDM sera/HDM-allergic sera, sera from HDM-sensitive individuals; control sera, sera from non-allergic individuals; HRP, horseradish peroxidase; IPTG, isopropyl-β-D-thiogalactopyranoside; PBST, phosphate-buffered saline containing 0.05% Tween 20; TBST, Tris buffered saline containing 0.05% Tween 20

Key words: house dust mite, recombinant allergen, recombinant Der f 1/2 fusion protein, capture immunoglobulin E-ELISA
Recombinant allergens are increasingly used in the diagnosis and treatment of allergic diseases, due to their high purity and consistency (9). Howard et al (10) defined the evolution of IgE responses to 112 recombinant or native allergen components during childhood, which may aid the identification of better diagnostic and prognostic biomarkers of allergic diseases. Mas et al (11) reported the use of the recombinant protein Salsola Kali in the diagnosis of allergic disease induced by Salsola kali. The combined expression of various antigens or major antigen epitopes as a fusion protein may increase the sensitivity of detection of antibodies targeted against allergens from a particular organism. He et al (12) engineered a recombinant antigen with epitopes from four hepatitis C viral fragments to aid the detection of anti-hepatitis C antibodies. Dai et al (13) constructed and overexpressed a fusion gene comprising three Mycobacterium tuberculosis antigen proteins; using the fusion polyprotein as an immunogen, multi-target antibodies were produced that exhibited significantly increased sensitivity for the clinical diagnosis of tuberculosis than mono-target antibodies reactive to the three respective antigens.

Omalizumab is a recombinant DNA-derived humanized IgG monoclonal antibody that suppresses allergic symptoms by binding to human IgE (14); thus, free IgE levels are reduced, preventing interactions between IgE and immune cells and decreasing the serum levels of inflammatory mediators (15). The detection of allergen-specific IgE is required for the diagnosis and management of IgE-mediated allergic disease. In the present study, two fusion allergens derived from the major allergenic HDM species Dermatophagoides farina (Der f1), Der f1 and Der f2, were cloned and expressed. Subsequently, a novel capture IgE-ELISA was developed using a recombinant Der f1/2 fusion protein (rDer f1/2), which was designed to improve the sensitivity of HDM allergen-specific IgE detection. The capture ELISA method involved the coating of wells with omalizumab to enrich serum IgE and reduce interference from IgG. Preliminary experiments were conducted using the assay to determine its reliability for accurate anti-allergen detection and test the potential of rDer f1/2 fusion protein-based ELISAs for the diagnosis of HDM-allergic disease.

Materials and methods

Serum samples, reagents and antibodies. All serum samples from HDM-sensitive individuals (HDM-allergic sera) and non-allergic individuals (control sera) were provided by The First Affiliated Hospital of Guangzhou Medical College (Guangzhou, China) between March 2013 and July 2015. A total of 28 subjects (13 males and 15 females, 18-55 years old) were enrolled in the present study. The patients were subjected to a skin prick test using dust mite allergen extract, serum samples were separated by centrifugation at 500 x g for 15 min at room temperature for the detection of IgE using the ImmunoCAP allergen detection system (Phadia AB; Thermo Fisher Scientific, Inc., Waltham, MA, USA). IgE levels were defined as follows: Level 3, 3.5-17.5 IU/ml; level 4, 17.5-50.0 IU/ml; level 5, 50.0-100 IU/ml and level 6, >100 IU/ml. The clinical-pathological characteristics of the patients are presented in Table I. A random cohort of 71 HDM-allergic serum samples (37 males and 34 females, 18-55 years old) were used to determine the sensitivity of capture IgE-ELISA and indirect IgE-ELISA. In total, 20 non-allergic individuals (8 males and 12 females, 18-55 years old) were used as negative controls. Ethical approval was obtained from The First Affiliated Hospital of Guangzhou Medical College and patients provided informed consent.

The restriction enzymes XhoI, NdeI and pET28 plasmid were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). E. coli BL21(DE3)pLysS cells were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). A Pierce™ 3′-diaminobenzidine (DAB) kit was purchased from Pierce (Thermo Fisher Scientific, Inc.). Biotinylated mouse anti-human IgE antibody (cat. no. 9160-08), streptavidin-labeled horseradish peroxidase (HRP; cat. no. 7100-05) and mouse anti-human IgE HRP-labeled antibody (cat. no. 9160-05) were purchased from SouthernBiotech (Birmingham, AL, USA).

Construction of the rDer f1/2 fusion expression vector. The pET28-Der f1 and pET28-Der f2 (Takara Biotechnology Co., Ltd.) expression vectors were constructed as previously described (16,17). Overlapping polymerase chain reactions were performed to splice the Der f1 and Der f2 genes. The construction of pET28-rDer f1/2 plasmid was confirmed by DNA Sanger sequencing (Sangon Biotech Co., Ltd., Shanghai, China). The pET28-Der f1/2 expression vector was engineered using NdeI and XhoI restriction sites. The fusion sequence encoding Der f1/2 was reported in GenBank (accession no. MF074325.1; https://www.ncbi.nlm.nih.gov/nuccore/MF074325.1). Der f1 amino acid residues 19-321 were linked to Der f2 residues 18-146 via a GGGS linker. The resultant recombinant protein contained a hexahistidine (6xHis) tag in its C-terminus.

Expression and purification of rDer f1, rDer f2 and rDer f1/2 proteins. In total, 5 µl recombinant plasmid pET28-Der f1/2 was transformed into 50 µl Escherichia coli BL21(DE3) pLysS cells using heat shock method (18). Cells were inoculated in Luria-Bertani medium (Merck KGaA, Darmstadt, Germany) containing 0.01 mg/ml kanamycin at 37°C for overnight. IPTG (1 mM)-induced expression was observed following growth for 3 h at 37°C, at which time cells were harvested by centrifugation at 8,000 x g for 2 min at 4°C. Pellets were resuspended in buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0) and the cells were lysed by ultrasonic homogenization. The supernatant and precipitate were collected and analyzed via SDS-PAGE (described below). Gels were stained with Coomassie G-250 Brilliant Blue for protein analysis as previously described (19). rDer f1, rDer f2 and rDer f1/2 in inclusion body fractions were solubilized with 6 M guanidine hydrochloride in 100 mM Tris (pH 8.0) for 2 h at room temperature (20). The proteins were purified by nickel affinity chromatography (Ni Sepharose 6 Fast Flow; cat. no. 17531801; GE Healthcare, Chicago, IL, USA) under denaturing conditions as previously described (21). Inclusion body surfaces frequently contain DNA, endotoxins and heteroproteins; washing with low concentrations of denaturing agent reduces impurities, thereby improving inclusion body protein purity (22). Deagglomeration of inclusion bodies into free loose structures with high concentrations of
Preparation of biotinylated Der f 1/2 fusion protein. rDer f 1/2 contained a 6xHis tag attached to its C-terminus. It was biotinylated using EZ-Link Sulfo-NHS-Biotin reagents (Thermo Fisher Scientific, Inc.), which enable simple and efficient molecular labeling (24). Fusion protein dissolved in PBS (pH 7.2) was incubated with 10 mM biotin reagent solution at room temperature for 30 min. Upon demonstration of protein labeling, the labeled fusion protein was purified by desalting column (HiTrap Desalting; GE Healthcare) in preparation for capture ELISA.

Indirect and capture IgE-ELISA. The IgE binding activities of recombinant proteins were detected by indirect and capture IgE-ELISAs. For indirect IgE-ELISA, 96-well plates were coated with recombinant antigen (100 ng/well, diluted in carbonate buffer, pH 9.6) at 4°C overnight. The plates were then blocked with 5% (w/v) DMS in PBS containing 0.05% Tween 20 (PBST) for 3 h at 37°C. Following washing, the plates were incubated with serum from HDM-allergic patients (1:5) for 2 h at 37°C, followed by incubation with mouse anti-human IgE biotin-labeled (1:2,000) for 1.5 h at 37°C. The plates were washed and subsequently incubated with streptavidin-HRP (1:4,000) for 30 min at 37°C. The plates were washed with PBST. Bound biotinylated-labeled antibody was detected by adding 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB; 1 mM); the reaction was stopped with 50 µl of H2SO4 (2 M). For capture IgE-ELISA, wells were coated with omalizumab (500 ng/well) overnight at 4°C. The wells were blocked with 5% DFM in PBS containing 0.05% Tween 20 for 3 h at 37°C and HDM-allergic human serum (1:5) was subsequently added to the wells prior to incubation for 2 h at 37°C. To select an optimal concentration, a standard serial dilution (50, 100, 200 and 400 ng/well) of biotinylated rDer f 1/2 was initially employed for 1 h at 37°C, 50 ng/well was selected for subsequent experiments. Each protein solution was incubated in wells with streptavidin-HRP for 0.5 h at 37°C, prior to addition of TMB. The TMB reaction was conducted for 10 min at 37°C prior to the application of H2SO4. For indirect and capture IgE-ELISAs, the absorbance was measured at 450 nm by a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. The experimental data were presented as the mean ± standard error of the mean. Data were analyzed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). Differences between groups were determined by analysis of one-way variance followed by Dunnett's t-test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Table I. Clinicopathological factors of the patients.

| Patient no. | Gender/age | HDM-specific IgE testa | Clinical history |
|-------------|------------|------------------------|----------------|
| 1           | F/45       | 3+                     | AD             |
| 2           | M/32       | 3+                     | AR             |
| 3           | F/18       | 3+                     | -              |
| 4           | F/50       | 3+                     | AD             |
| 5           | M/53       | 3+                     | BA             |
| 6           | M/34       | 3+                     | -              |
| 7           | F/31       | 3+                     | BA             |
| 8           | F/55       | 4+                     | AR + BA        |
| 9           | M/42       | 4+                     | AR             |
| 10          | F/24       | 4+                     | AD             |
| 11          | F/37       | 4+                     | BA             |
| 12          | F/52       | 4+                     | BA             |
| 13          | M/18       | 4+                     | -              |
| 14          | M/29       | 4+                     | AR             |
| 15          | F/35       | 5+                     | -              |
| 16          | M/37       | 5+                     | AD             |
| 17          | F/28       | 5+                     | AR             |
| 18          | M/53       | 5+                     | AD + AR        |
| 19          | F/48       | 5+                     | AR             |
| 20          | F/21       | 5+                     | AD             |
| 21          | M/46       | 5+                     | BA             |
| 22          | F/33       | 6+                     | -b             |
| 23          | M/41       | 6+                     | AR             |
| 24          | F/54       | 6+                     | AD + BA        |
| 25          | M/34       | 6+                     | AR             |
| 26          | M/21       | 6+                     | AD             |
| 27          | F/44       | 6+                     | BA             |
| 28          | F/27       | 6+                     | AD + AR        |

aIgE levels were determined by the ImmunoCAP system with Dermatophagoides farina antigen. b, data not shown. IgE levels were defined as follows: Level 3, 3.5-17.5 IU/ml; level 4, 17.5-50.0 IU/ml; level 5, 50.0-100 IU/ml and level 6, >100 IU/ml. AR, allergic rhinitis; AD, atopic dermatitis; BA, bronchial asthma; HDM, house dust mite; Ig, immunoglobulin.

guanidinium hydrochloride enables the material to be stored in buffer in a soluble state. The solution was treated with 20 mM β-mercaptoethanol for 30 min prior to diluting renaturation. Denatured proteins were refolded and diluted 10-fold with 0.5 M L-arginine (pH 8.0) (23). Arginine buffer was added to the denatured protein solution slowly (flow rate of 0.5 ml/min) and incubated overnight at 4°C. The solution was then dialyzed for 24 h using buffer containing 20 mM Tris and 150 mM NaCl. Following renaturation, protein concentrations were determined by the Bradford method.

IgE-western blotting and IgE-dot blotting. In total, 20 µg of rDer f 1/2 fusion protein was loaded in each well. Proteins were separated by 12% SDS-PAGE and then transferred to polyvinylidene difluoride membranes for western blotting. For dot blotting, 2 µl of the allergens (rDer f 1, rDer f 2 and rDer f 1/2) was separately spotted onto nitrocellulose membranes at a concentration of 1 µg/µl. The membranes were blocked with 5% Difco™ Skim Milk (DSM; BD Biosciences, San Jose, CA, USA) diluted in TBS containing 0.05% Tween 20 (TBST) at 4°C overnight. Serum samples from 15 HDM-allergic patients and 15 control subjects were also analyzed and incubated for 2 h at 37°C. Subsequently, mouse anti-human IgE-Fc-HRP antibody was applied (1:2,000 in TBST with 1% DSM) for 1 h at 37°C. Antibody-antibody complexes on membranes were visualized using a Pierce™ DAB kit.
Results

Linear IgE B cell epitopes of Der f 1 and Der f 2. B cell epitopes are antigenic regions in proteins recognized by B cells and are considered to be important for the diagnosis of allergies via the serum (25). The literature was investigated to identify linear IgE B cell epitopes in Der f 1 and Der f 2; it was revealed that Der f 1 (GenBank accession no. EF139428.1) and Der f 2 (GenBank accession no. FJ436110) separately possess three B cell epitopes. The amino acid sequences of the epitopes in Der f 1 and Der f 2 (26-28) are presented in Fig. 1. It was hypothesized that Der f 1/2, a fusion protein comprising these B cell epitopes, would bind with a broader range of specific IgEs, rendering it suitable for the diagnosis of HDM allergy.

Expression, purification and IgE binding activity of rDer f 1/2. A schematic diagram of the rDer f 1/2 construct is presented in Fig. 2A. The rDer f 1/2 protein was expressed in the form of inclusion bodies, solubilized with guanidine hydrochloride and identified by SDS-PAGE (Fig. 2B). Purification of rDer f 1/2 by nickel affinity chromatography resulted in the presence of a single band migrating at a theoretical molecular weight of 46 kDa (Fig. 2C). IgE western blot analysis revealed that rDer f 1/2 bound to IgEs when incubated with serum samples from 15 patients with diagnosed HDM allergies, but did not bind to IgEs in sera obtained from the 15 non-allergic control subjects (Fig. 2D). These results indicated that rDer f 1/2 exhibited a selective, strong binding affinity for IgE in HDM-allergic sera.

Enhanced sensitivity of capture IgE-ELISA based on rDer f1/2. Schematic diagrams of the capture and indirect IgE-ELISA
methods employed for the detection of HDM-specific IgEs are presented in Fig. 4A and B, respectively. A preliminary assay performed to optimize the rDer f 1/2-biotin conjugate concentration used during the assay revealed that 0.5 µg/ml was optimal for capture IgE-ELISA, based upon the relative OD values following incubation with HDM-allergic and control sera (Fig. 5A).

Subsequently, the relative sensitivity of rDer f 1/2-based capture IgE-ELISA compared with indirect IgE-ELISA for the detection of specific IgEs was determined using HDM-allergic serum samples containing various levels of IgE (as defined by ImmunoCAP). Significantly increased OD values were reported following capture IgE-ELISA compared with indirect IgE-ELISA using level 4 and 5 serum samples (P<0.05; Fig. 5B).

Finally, a random cohort of 71 HDM-allergic serum samples was used to determine the specific IgE detection reliability of the two ELISA methods. The mean OD value for rDer f 1/2-specific IgE detected by capture IgE-ELISA was significantly increased compared with indirect IgE-ELISA; indirect and capture ELISAs yielded mean OD values of 1.754±0.25 and 2.52±0.23, respectively (P<0.01; Fig. 5C). The P/N value was defined as the ratio of the optical density values of positive samples compared with the negative samples. The positive rates for specific IgE detection in the random HDM-allergic serum cohort were 68/71 (95.8%) for indirect IgE-ELISA and 71/71 (100%) for capture IgE-ELISA when the cutoff was set to P/N value >2.1 (Table III). Collectively, the results suggested that capture IgE-ELISA based on rDer f 1/2 protein exhibits good sensitivity for HDM allergen-specific IgEs.

**Discussion**

The sensitivity of indirect ELISA for the detection of allergen-specific IgE is decreased by competition from high-titer IgG, reducing the accuracy of indirect ELISAs in determining the levels of allergen-specific IgE in the sera of patients (30). To overcome this limitation, capture ELISA was employed, using anti-IgE antibodies to capture total IgE antibodies in sera, thereby enhancing sensitivity for the detection of allergen-specific IgEs (31). In a random HDM-allergic serum cohort, positive rDer f 1/2-specific IgE detection rates of 68/71 (95.8%) and 71/71 (100%) were reported with indirect and capture ELISA, respectively, demonstrating the increased sensitivity of capture IgE-ELISA for allergen-specific IgE detection compared with indirect IgE-ELISA.

The sensitivity of the capture ELISA method reported in the present study was enhanced by the high efficiency of the rDer f 1/2 fusion protein in detecting IgE. Antigen coatings
produced from HDM extract mixtures exhibit low sensitivity due to the inclusion of very small amounts of effective allergen components. Compared with natural antigen extraction, recombinant antigens possess the benefits of high antigenic consistency and low risk of contamination by impurities (32). Recombinant allergens mimic the properties of natural allergens or modified variants; however, they can be manipulated to improve safety or efficacy (33).

It was previously reported that Der f 1- and Der f 2-specific IgEs were detected in 86.2 and 89.4% of patients with HDM-sensitive respiratory allergy, respectively, whereas IgEs that bound to Der f 1 and Der f 2 were detected in 92.3% of the patient population (33). Multi-allergen fusion proteins produced by genetic engineering are a novel strategy for the diagnosis or treatment of allergies.

Allergen fusion proteins enable various allergen components to be combined into a single molecule, thereby simplifying molecular diagnostics. The development of a recombinant molecule comprising four major dog allergens for the diagnosis and vaccination of patients with dog allergies has been reported (34). Additionally, the two major allergens associated with Japanese cedar pollen were expressed as a fusion protein and conjugated to polyethylene glycol to improve solubility and create a safer vaccine (35). These advancements promote the replacement of cruder allergen extract mixtures with recombinant allergen proteins for the diagnosis of allergic diseases.

Table III. Positive rate of IgE-ELISA for sera from patients with HDM-allergic disease as determined using indirect-ELISA and capture-ELISA.

| Methods       | Positive rate [cutoff (P/N>2.1)] (%) | Positive rate (cutoff=0.7)* (%) |
|---------------|-------------------------------------|--------------------------------|
| Indirect ELISA| 68/71 (95.8)                        | 56/71 (78.9)                   |
| Capture ELISA | 71/71 (100)                         | 66/71 (93.0)                   |

* Artificial value for comparison. P/N, ratio of the optical density values of the positive samples compared with the negative samples. HDM, house dust mite; Ig, immunoglobulin.
The findings of the present study require further validation with increased sample sizes. Additionally, the efficacies of rDer f 1/2 and the capture IgE-ELISA method were only investigated compared with the fusion proteins Der f 1 and Der f 2, antigens from two highly dominant HDM allergen groups. The high sensitivity of the capture IgE-ELISA method reported in the present study indicated that it may be particularly useful for identifying minor allergens, such as HDM allergens from groups beyond groups 1 and 2. The sensitivity of this method for the detection of minor allergens requires further investigation.

In conclusion, rDer f 1/2 was successfully expressed and purified, and was demonstrated to exhibit high IgE-binding activity in HDM-allergic sera. The novel capture IgE-ELISA based upon the biotinylation of Der f 1/2 exhibited increased efficacy for the detection of HDM allergen-specific IgEs compared with indirect IgE-ELISA. Thus, it was demonstrated to be a reliable method for the accurate detection of specific anti-allergen IgEs in allergic sera. Further studies employing this method may aid to improve the diagnosis of HDM-induced allergic disease and the development of anti-allergy vaccines.

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Availability of data and materials
All data generated or analyzed in the present study are available from the corresponding author upon reasonable request.

Authors' contributions
ZZ performed the experiments and drafted the manuscript. ZC, YiH, JH, and YoH participated in the experiments. JC and KJ made substantial contributions to the design of the present study and wrote the manuscript. All the authors reviewed and approved the final version of the manuscript.

Ethics approval and consent to participate
Permission to conduct the present study was obtained from the Ethics Committee of The First Affiliated Hospital of Guangzhou Medical College (Guangzhou, China). All procedures involving human participants were in accordance with the ethical standards of the committee. Informed consent was obtained from all participants.

Patient consent for publication
All procedures involving human participants were in accordance with the ethical standards of the committee.

Competing interests
The authors declare that they have no competing interests.

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