

Development of time-resolved immunofluorometric assays for the detection of house dust mite-allergic IgE in human sera

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ABSTRACT

Dermatophagoides farinae and *D. pteronyssinus* are the prevalent house dust mites (HDM) in tropical countries and are associated with allergic diseases. This investigation developed a time-resolved immunofluorometric assay (TR-IFMA) for the first time to detect specific IgE antibody in patients with skin prick test positive to HDM but no detectable IgE by other means. Levels of IgE to natural and recombinant HDM allergens were measured by TR-IFMA in 50 HDM-allergic patients and 19 healthy participants compared to sandwich enzyme-linked immunosorbent assay (ELISA). A recombinant allergen, rDerf2, showed a 14 kDa band corresponding to broad range proteins of natural HDM. TR-IFMA showed sensitivity lower than 0.35 kUA/l. TR-IFMA employing three HDM antigens showed good correlations with sandwich ELISA at R2 0.93-0.96. TR-IFMA detected HDM IgE in 62, 62, 25 percent of allergic patient serum sample compared to 28, 32, and 22 percent detected by ELISA result using three HDM allergen. TR-IFMA also detected 26.3, 31.6, and 5.3 percent positive samples from 19 healthy participants while ELISA showed 0, 5.3, and 0 percents IgE positive samples. The use of rDerf2 as an HDM allergen for the assay was verified with no statistically different from other HDM allergens. TR-IFMA showed lower detection limit than ELISA and yielded higher sensitivity for serum of people with allergic symptoms with no detectable HDM IgE. It is anticipated that TR-IFMA for HDM-specific IgE detection will play an important role in future diagnosis of HDM allergy in clinical laboratories and for

different research purposes.

Keywords: House Dust Mites; Time-Resolved Immunofluorometric Assay; Allergy; IgE

1. INTRODUCTION

House-dust mites (HDMs) represent one of the most important allergen sources for the development of allergic diseases worldwide, particularly asthma and allergic rhinitis [1]. *Dermatophagoides farinae* (Df, originally known as American HDM) and *Dermatophagoides pteronyssinus* (Dp, European HDM) are the predominant species in tropical and subtropical climates [2]. More than 80% of allergic patients are sensitized to mite allergens [3,4].

Positive skin prick tests (SPT) or serum IgE antibodies to HDMs are demonstrable in allergic patients. However, if patients are allergic to histamine, skin prick test could lead to complicated immunoreaction harmful to the patients [5]. Enzyme-linked immunosorbent assay (ELISA) is the method used to quantify serum HDM-IgE *in vitro* in laboratories [6]. However, a more sensitive *in vitro* test is needed for people who have dust mite allergic clinical diagnosis but shown undetectable HDM-IgE in their serum.

Monoclonal antibodies (mAbs) have been frequently used in allergy research. Their uses include the quantification of environmental allergen [7], allergens purification [8], and crystal structure [9]. Specificity for unique epitopes and unlimited *in vitro* production capability are the advantage of allergen-specific mAbs.

The purpose of this study was to development a time-resolved immunofluorometric assay (TR-IFMA) for the measurement of HDM-specific IgE. The TRF is characterized by lower detection limits and greater specificity,

reproducibility and practicability [10]. Two group II HDM allergens and a group two recombinant vector were used for validation of the assay.

2. MATERIALS AND METHODS

2.1. Reagents

Commercial allergens were purchased from Allertech (ALK, UK). DELFIA[®] Wash, DELFIA[®] Assay Buffer, DELFIA[®] EU-labeled Streptavidin and DELFIA[®] Enhancement Solution were purchased from Perkin-Elmer Life Sciences (Wallac Oy, Turku, Finland). Monoclonal anti-group 2 antibodies (mAb 1D8 clone, Indoor biotechnologies Ltd, Manchester, UK). Biotinylated rabbit-anti mouse IgG solution and Biotinylated-labeled Mouse anti-human IgE antibody purchased from SouthernBiotech (Birmingham, USA). BCIP/NBT 1 Component Substrate and ABTS[®] Peroxidase 1 Component substrate Solution were purchased from KPL (MD, USA). C96 MicroWell[®] plates were purchased from Nunc[™] (Roskilde, Denmark). The instrument used for measurement - DELFIA 1420 Automatic Immunoassay System - is the product of Perkin-Elmer Life Sciences (Wallac Oy, Turku, Finland).

2.2. HDM Extracts and Recombinants Mite Allergens

Total HDM extracts of *D. farinae* (Df) and *D. pteronyssinus* (Dp) were prepared from Spent Mite medium from Siriraj Dust Mite Center, Bangkok, Thailand [7]. The extracts were stored at -20°C.

Recombinant rDerm2 allergen was constructed with ligated cDNA in pPICZ vector. It was a kind gift from Assist. Prof. Dr. Surapon Piboonpocanun, the Institute of Molecular Biology and Genetics, Mahidol University, Thailand. The positive transformants were subjected to expression in *Pichia pastoris* [11,12]. The supernatants were stored at -20°C until used.

Proteins were separated by SDS-PAGE followed by transferring the fractionated proteins to a nitrocellulose membrane using method as described by the manufacturer with a wet blotting apparatus (Bio-Rad, USA). The blotted membrane was blocked overnight at 4°C. The membrane was further incubated with Monoclonal anti-group 2 antibodies, followed by Biotinylated rabbit-anti mouse IgG solution (1:5000) and BCIP/NBT 1 Component Substrate.

2.3. Immunological Analysis

2.3.1. Enzyme-Linked Immunosorbent Assay (ELISA)

Specific IgE to Df, rDerm2 and Dp were analyzed by

the ImmunoCAP[™] system by using the cut-off limit 0.35 kilo units of aprotinin-specific antibodies per litre (kU_A/l), as recommended by the manufacturer. They were analyzed by sandwich ELISA (will be referred to as ELISA thereafter) as previously described [13]. Wells were coated with 1D8 clone mAb and let incubated over-night. Then 1 ng/well allergens in PBS containing 1% BSA were added. The wells were further incubated with serum (1:10), followed by biotinylated-labeled Mouse anti-human IgE antibody (1:1000), streptavidin-peroxidase and ABTS[®] Peroxidase 1 Component substrate. The result was measured in a DELFIA 1420 Automatic Immunoassay System.

2.3.2. Time-Resolved Immunofluorometric Assay (TR-IFMA)

Specific IgE to all three allergens were analyzed by the ImmunoCAP[™] system by using the cut-off limit 0.35 kU_A/l as recommended by the manufacturer. Df, recombinant Derm2 and Dp were analyzed by TR-IFMA [13]. Wells were coated with mAb and incubated over-night. After incubation, 1 ng/well allergens in PBS containing 1 % BSA were added. The wells were further incubated with serum (1:10), followed by biotinylated-labeled Mouse anti-human IgE antibody (1:1000), DELFIA[®] EU-labeled Streptavidin and DELFIA[®] enhancement solution. The result was measured in a DELFIA 1420 Automatic Immunoassay System.

2.4. Patients and Collection of Samples

Fifty rhinitis patients with/without intermittent or persistent, mild-to-moderate asthma with positive result to Skin Prick Test (SPT) were selected for this investigation by Dr. Nares Wongpitoon or Dr. Voravan Durongpisitkul of Department of Pediatric, Police General Hospital, Bangkok, Thailand. As the control group, 19 healthy subjects with no history of allergic diseases were included. The study was approved by the Ethics committee of the Police general Hospital and written informed consent was obtained from all participants.

Five milliliter of venous blood was taken from each participant. The blood was centrifuged within 6 hr of collection at 400 g for 30 min. The serum was then collected and stored frozen at -20°C.

In order to quantify specific IgE levels, serum from three highest HDM-sensitized individuals as analyzed by ELISA and TR-IFMA were analyzed by the ImmunoCAP[™] system at the laboratory of Faculty of Medicine, Mahidol University, Bangkok, Thailand. The IgE levels in several dilutions of the reference serum were analyzed. Fifth calibrator points in two-fold dilutions ranging 0.22-3.49, 0.22-3.49 and 0.28-4.55 kU_A/l of Df, rDerm2 and Dp specific IgE respectively were made for use in

calibrating curve of both ELISA and TR-IFMA.

2.5. Statistical Analysis

Levels of specific IgE to HDM allergens in ELISA and TR-IFMA were obtained from Version 13 (Chicago, IL, USA).

3. RESULTS

3.1. HDM-Allergen Characteristics

HDM-allergen characteristics were determined by SDS-PAGE and Immunoblot using monoclonal antibody, as shown in **Figure 1**. Result from SDS-PAGE showed the MW of Df and Dp ranged from 10 to 72 kDa, while the molecular weight of rDerf2 was approximately 14 kDa. Immunoblot also confirmed rDerf2 molecular weight at 14 kDa.

3.2. Calibration Curve and Detection Limit

A typical standard curve for HDM-specific IgE using ELISA and TR-IFMA is shown in **Figure 2**. The sensitivity (± 2 standard deviations) of the assay, as calculated from X replicates of the zero standards, was about $< 0.35 \text{ kU}_A/\text{l}$. The calibration curve was linear.

3.3. Subject Characteristics

Specific antibodies to HDM allergens were examined in a total of 69 subjects. Subjects were screened by SPT using commercial Der f, Der p, CR, Grass, Dog, Cat, Wheat, Shrimp, Feather, M1 and M2. The demographic clinical characteristics of the subjects were shown in **Table 1**.

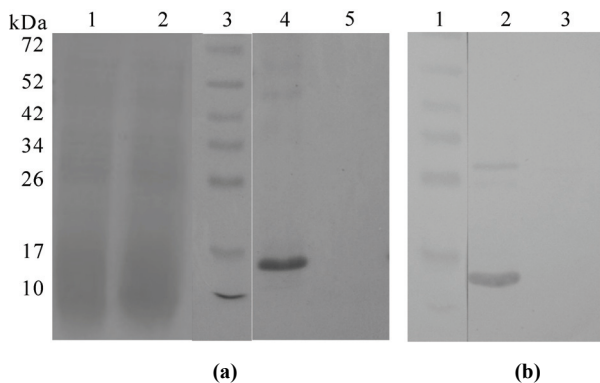


Figure 1. The protein components of HDM allergen were determined by SDS-PAGE (a); Lane 1: Df; Lane 2: Dp; Lane 3: Molecular mass standards (in kDa); Lane 4: rDerf2; Lane 5: pPICZ. (b) Immunoblot Lane 1: Molecular mass standards (in kDa); Lane 2: rDer f02; Lane 3: pPICZ.

3.4. Comparison of TR-IFMA and ELISA

Number of positive samples from ELISA and TR-IFMA in allergic patient and healthy participant serum as seen using Df, rDerf2 and Dp allergen against group II mAb is shown in **Table 2**. TR-IFMA detected HDM IgE in higher percentage of allergic patient serum sample when tested using Df, rDerf2 and Dp allergen (62, 62, 25 percent) compared to ELISA result (28, 32, and 22 percent respectively). In 19 healthy subjects, ELISA detected HDM IgE in 0, 5.3, and 0 percents samples tested by the three HDM allergens while TR-IFMA found 26.3, 31.6, and 5.3 percent positive samples respectively.

TR-IFMA was compared to ELISA by parallel testing of 50 serum specimens from rhinitis patients utilizing Df, rDerf2 and Dp allergen respectively. Among allergic patients very good overall correlation was found (**Figure 3**). The linear regression coefficient values calculated from the positive specimens varied from 0.93-0.96.

Table 1. Demographic and characteristics of the study individuals.

Characteristics	Groups	
	allergic individuals (A)	non-allergic individuals (B)
Number of subjects (n)	50	19
Age (years, mean \pm SD)	39.52 \pm 9.93	25.21 \pm 6.9
Gender (male/female)	24/26	4/15
Clinical diagnosis (N, %)	50, 100 %	N/A
Catagorized by allergen (%)		N/A
Der f	100 %	
Der p	100 %	
CR	20 %	
Grass	8 %	
Dog	4 %	
Cat	8 %	
Wheat	0 %	
Shrimp	0 %	
Feather	0 %	
M1	12 %	
M2	12 %	

Der p, *Dermatophagoides pteronyssinus*; Der f, *Dermatophagoides farinae*; CR, American Cockroach; Grass, Bermuda grass pollen; Dog, Dog epithelium; Cat, Cat pelt; Feather, feather of Chicken, Duck, and Goose; M1, *Penicillium notatum*, *Aspergillus niger*, *Drechslera*, *Cladosporium sphaerospermum*, *Alternaria*; M2, *Curvularia*, *Fusarium*, *Aureobasidium*, *Mucor*, *Rhizopus*

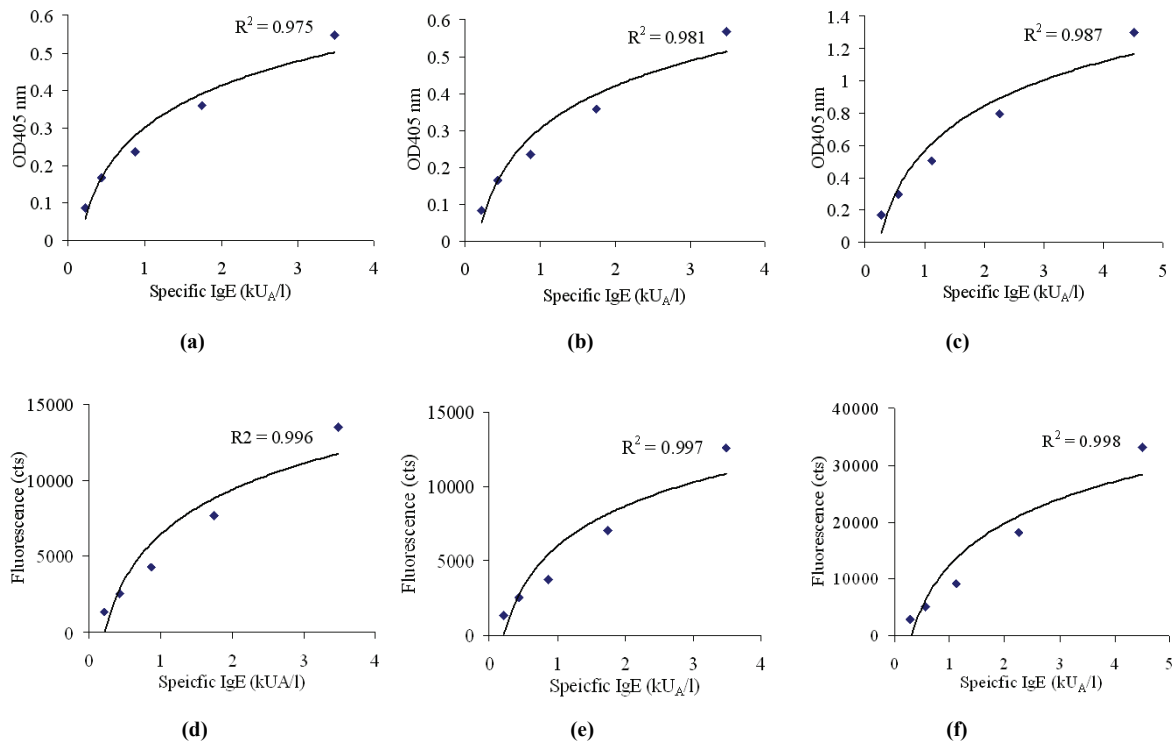


Figure 2. Standard curve for sandwiched ELISA for (A) Df, (B) rDerf2, (C) Dp. Standard curve for TR-IFMA for (D) Df, (E) rDerf2, (F) Dp. Each point on the standard curve is the mean of 3 duplicate measurements.

Table 2. Number of positive samples from ELISA and TR-IFMA in allergic patient and healthy participant serum as seen using Df, rDerf2 and Dp allergen against group II mAb.

Allergen tested	Number of positive samples: n (frequency %)	
	Allergic patients (A) Total n = 50	Healthy participants (B) Total n = 19
Df		
ELISA	14 (28)	0 (0)
TR-IFMA	31 ¹ (62)	5 (26.3)
rDerf2		
ELISA	16 (32)	1 (5.3)
TR-IFMA	31 ² (62)	6 (31.6)
Dp		
ELISA	11 (22)	0 (0)
TR-IFMA	25 ³ (50)	1 (5.3)

^{1,2} and ³ correspond to the same data in **Table 3**.

TR-IFMA positive cases from serum of allergic patients tested with all three HDM allergen were separated into two groups, namely those from ELISA positive and ELISA negative samples. Number of samples, frequency

and median of each group is shown according to specific allergen in **Table 3**. Median, standard deviation, 25 and 75 percent values are presented in **Figure 4**. Among TR-IFMA positive samples, 54.84, 48.39, and 56 percents of Df, rDerf2, and Dp were identified as ELISA negative samples.

Concentrations of HDM IgE TR-IFMA from all three HDM allergens testing (allergic patients and healthy participants) were subjected to test for several related sample using Friedman test. At N =69, *p* value equals 0.093. The use of any of the three HDM allergens does not give a statistically significant result in TR-IFMA technique.

4. DISCUSSIONS

It has been reported that *D. farinae* which homologous with *D. pteronyssinus* is composed of broad range of 10 to 72 kDa. In the present study, Immunoblotting analysis of rDer f 2 using a specific mAb showed one band of 14 kDa (**Figure 1**), agreeing with previous reports and indicating that rDer f 2 corresponded to *D. farinae* [14,15]. It is known that *D. farinae* usually coexists with *D. pteronyssinus*. As a result the assay measuring IgE binding to Derf2 would not distinguish *D. farinae* sensitization with cross reactivity [16].

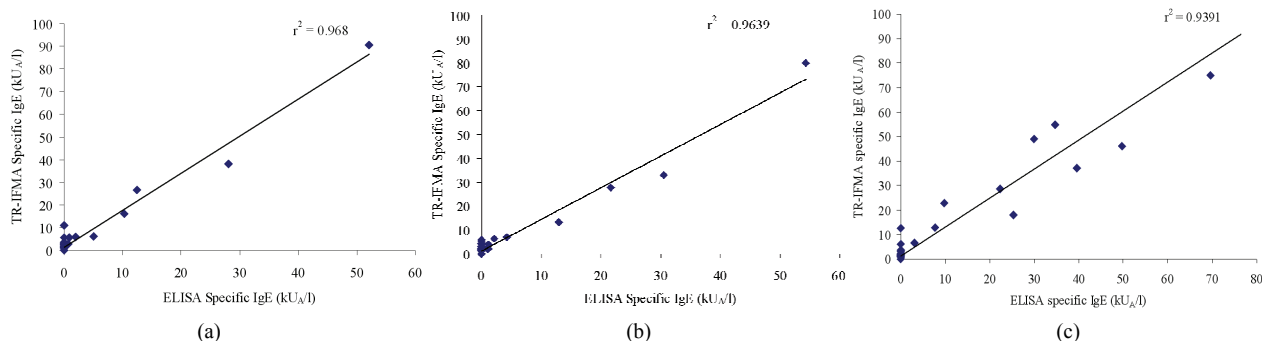


Figure 3. Correlation between levels of specific IgE against Df (a), rDerf2 (b) and Dp (c) measured by ELISA and TR-IFMA.

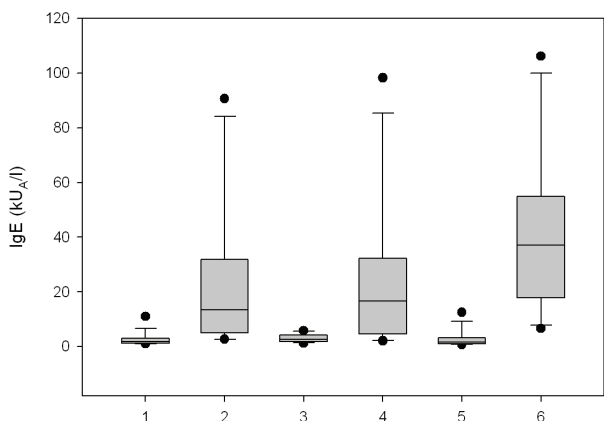


Figure 4. TR-IFMA positive cases from serum of allergic patients tested with all three HDM allergens. Median, standard deviation, 25 and 75 percent values are presented. HDM allergens Df: 1) ELISA negative 2) ELISA positive rDerf2: 3) ELISA negative 4) ELISA positive Dp: 5) ELISA negative 6) ELISA positive.

Table 3. TR-IFMA positive cases in serum from allergic patients categorized by ELISA result in Df, rDerf2 and Dp testing.

HDM allergens	TR-IFMA positive cases in symptomatic patients	
	ELISA positive	ELISA negative
Df: total 31 samples ¹		
n (frequency %)	14 (45.16)	17 (54.84)
Median (kU _A /l)	13.37	1.81*
rDerf2: total 31 samples ²		
n (frequency %)	16 (51.61)	15 (48.39)
Median (kU _A /l)	16.67	2.74*
Dp: total 25 samples ³		
n (frequency %)	11 (44)	14 (56)
Median (kU _A /l)	37.19	1.66*

Mann-Whitney Rank Sum Test; *p* < 0.001*

4.1. Levels of Specific IgE Reactivity to HDM Allergens: Lower Detection Limit and Increase Testing Sensitivity

The IgE reactivity profile for the sera from allergic patients (n = 50) and healthy participants (n = 19) was determined by three different allergens, namely Df, rDerf 2 and Dp measured by TR-IFMA compared with ELISA.

As shown in **Table 2**, the efficacy of recombinant antigen (rDerf 2) and natural antigen (Df or Dp) were examined in allergic patients and non-allergic individuals by both TR-IFMA and ELISA. In allergic patients, we found that only 28, 32, and 22 percents result was obtained by ELISA, while 62, 62, and 50 percent of patient serum reacted with Df, rDerf2, and Dp respectively using TR-IFMA. The increase in rate of HDM IgE detection was significant at 34, 30 and 28 percents for all three allergen respectively. This is the first indication

suggesting that TR-IFMA of all three HDM allergens show higher sensitivity for serum of people with allergic symptoms with no detectable HDM IgE. In addition, when ELISA showed 0, 5.3, and 0 percent positive samples in healthy participant serum, TR-IFMA detected 26.3, 31.6, and 5.3 percent positive samples. That is the 26.3, 21.3 and 5.3 percent higher detection rate across the board.

In 50 HDM-SPT positive allergic subjects, 48 percents of this group showed cross-reactive IgE binding to all three allergens measured by TR-IFMA, while only 22 percent showed cross-reactive IgE binding to all three allergens measured by ELISA. Additionally, cross-reactive IgE to two allergens was also observed by both TR-IFMA and ELISA. TR-IFMA showed 10, 2 and 0 percent cross-reactivity between Df/rDerf2, rDerf2/Dp and Df/Dp, while ELISA result showed 2, 0, and 0 percent respectively. In healthy participants there was no cross-reactivity report over all three antigens. Only 15.8 and 5.3

percent cross-reactivity were reported for Df/rDerf2 and rDerf2/Dp. These numbers confirm TR-IFMA sensitivity over ELISA, and suggests that rDerf2 is a slightly better antigen as seen from cross-reactivity data.

Table 3 and **Figure 4** revealed lower detection limits and the sensitivity of TR-IFMA. All HDM allergens provided significantly different median between ELISA positive and negative samples in Mann-Whitney Rank Sum Test with $p < 0.001$. **Figure 4** shows that even though ranges of all three ELISA negative groups showed overlap with the ELISA positive ones, one hundred percent of all three ELISA negative populations stayed within 40 percentile of the corresponding ELISA positive population. TR-IFMA was able to show real value of some ELISA negative samples beautifully in this demonstration.

This investigation employed three different HDM allergens, namely Df and Dp from natural sources, and a recombinant rDerf2. Result from the test for several related sample using Friedman test showed that TR-IFMA result using all three allergens was not significantly different from one another. Thus this investigation suggests that rDerf2 can be used as a HDM allergen in TR-IFMA. Since rDerf2 can be expressed in large quantity, this should ensure a homogeneous batch supply of HDM allergen for time to come.

Immunoassays based on time-resolved fluorometry represents an attractive option that offers several advantages over other traditional techniques, including very high sensitivity, no use of radioactive reagents, stability of the reagents, low background interference and a wide test range [17, 18]. Additionally, the use of a specific mAb as capture antibody in this study avoided over estimation of allergens by reducing the cross-reactive epitopes recognition. The use of specific polyclonal serum avoided the loss of detection produced by conformational changes affecting one or more epitopes. Hence, a specific anti-HDM polyclonal serum has been used as the secondary antibody in this investigation, unlike most of other immunoassays described [19].

The time-resolved immunofluorometric assay developed in this investigation provides sensitive and highly specific techniques for the determination of HDM-specific IgE in human sera. Measurements achieved with TR-IFMA and ELISA were highly correlated (R^2 between 0.93-0.96). In regard to the limit of detection, the assay allows the detection of HDM-specific IgE as low as $< 0.35 \text{ kU}_A/\text{l}$, which is well below limits of detection reported by other techniques (Immunoblot, ELISA, RIA); TR-IFMA can therefore be considered as the most sensitive method for quantifying HDM-specific IgE. In addition, the assay ranges of TR-IFMA is the wider than those of ELISA in our laboratory. Our data was consis-

tent with previous reports [20,21]. It is clearly seen that TR-IFMA showed sensitivity to serum of allergic patients with symptom whose serum were formerly HDM IgE negative using ELISA. The analysis of real samples from HDM positive allergic patients compared to HDM negative but positive to aeroallergen demonstrated that a broad range of HDM-specific IgE could be detected with the present techniques.

5. CONCLUSIONS

TR-IFMA was developed for HDM allergen IgE testing and evaluated. Such improvements may be useful for screening of HDM allergy and other aeroallergens. TR-IFMA of all Df, rDerf2 and Dp showed lower detection limit than ELISA and yielded higher sensitivity for serum of people with allergic symptoms with no detectable HDM IgE. Recombinant HDM allergen, rDerf2, was proven to be as good an allergen as its natural counterpart, namely Df and Dp. It is anticipated that TR-IFMA for HDM-specific IgE detection will play an important role in future diagnosis of HDM allergy in clinical laboratories and for different research purposes.

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