DETECTION OF IGE AGAINST MITE ALLERGENS USING TIME-RESOLVED FLUOROIMMUNOASSAY

By
Ratchanoo Phiphatchaipaisarn

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
MASTER OF SCIENCE
Department of Biology
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การตรวจวัดระดับ IgE ต่อสารก่อภูมิแพ้ไรฝุ่นด้วย time resolved fluoroimmunoassay

โดย

นางสาวรัชนู พิพัฒน์ชัยไพศาล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ภาควิชาชีววิทยา

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The Graduate School, Silpakorn University has approved and accredited the Thesis title of "Detection of IgE Against Mite Allergens using Time-Resolved Fluoroimmunoassay" submitted by Miss Ratchanoo Phiphatchaipaisarn as a partial fulfillment of the requirements for the degree of Master of Science in Biology

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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 sandwich time-resolved fluoroimmunoassay (sandwichTRF) 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 native and recombinant HDM allergens were measured by TRF in 50 HDM-allergic patients and 19 healthy subjects compared to sandwich enzyme-linked immunosorbent assay (sandwichELISA). A recombinant allergen, rDer f 2, showed a 14 kDa band corresponding to broad range proteins of native HDM. sandwichTRF employing of Df, rDer f 2 and Dp showed good correlations with sandwichELISA at r^2 0.93-0.96. HDM IgE of Df, rDer f 2 and Dp detected from sandwichTRF are 62 %, 62 % and 50 % of allergic patient serum sample compared to 26 %, 32 % and 22 % detected from sandwichELISA, respectively. Sandwich TRF also detected 26.3 %, 31.6 % and 5.3 % positive samples from 19 healthy subjects while sandwichELISA showed 0 %, 5.3 % and 0 % IgE positive samples. The use of rDer f 2 as an HDM allergen for the assay was verified with no statistically different from other HDM allergens. Although with lower detection limit than sandwichELISA but sandwichTRF yielded higher sensitivity for patient sera with allergic symptoms but no detectable HDM IgE. It is indicated that TRF for HDM-specific IgE detection could play an important role in future diagnosis of HDM allergy.

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ไรฝุ่น *Dermatophagoides farinae* และ *D. pteronyssinus* เป็นไรฝุ่นบ้านที่พบเห็นได้บ่อย ๆ ในประเทศเขตร้อนชื้น และเป็นสาเหตุสำคัญของการก่อให้เกิดโรคภูมิแพ้ ในการศึกษาครั้งนี้เป็นการพัฒนาวิธี sandwich time-resolved fluoroimmunoassay (sandwichTRF) เพื่อตรวจวัดแอนติบอดี IgE ในผู้ป่วยที่ให้ผลบวกด้วยวิธี skin prick test แต่ไม่สามารถตรวจวัดได้ด้วยวิธีอื่น ๆ โดยใช้ซีรัมจากผู้ป่วยโรคภูมิแพ้ 50 คน และจากผู้ที่มีสุขภาพแข็งแรง 19 คน แล้วเปรียบเทียบกับผลการตรวจด้วยวิธี sandwich enzyme-linked immunosorbent assay (sandwichELISA) ผลการศึกษาปรากฏว่า โปรตีนสังเคราะห์ rDer f 2 มีขนาดโปรตีน 14 kDa เท่ากับโปรตีนสารก่อภูมิแพ้ในธรรมชาติ ระดับแอนติบอดี IgE ที่วัดด้วยวิธี sandwichTRF ต่อโปรตีนสารก่อภูมิแพ้ของไรฝุ่น Df, rDer f 2 และ Dp มีค่าความสัมพันธ์กับวิธี sandwichELISA (r^2 0.93-0.96) จากการเปรียบเทียบพบว่า วิธี sandwichTRF สามารถหาค่าแอนติบอดี IgE ต่อ Df, rDer f 2 และ Dp ได้ที่ระดับ 62, 62 และ 50 เปอร์เซนต์ของผู้ป่วยโรคภูมิแพ้ ส่วนวิธี sandwichELISA วัดได้เพียง 28, 32 และ 22 เปอร์เซ็นต์ตามลำดับ สำหรับผู้ที่มีสุขภาพแข็งแรง 19 คน วิธี sandwichTRF สามารถตรวจหาค่าแอนติบอดี IgE ต่อ Df, rDer f 2 และ Dp ได้ที่ระดับ 26.3, 31.6 และ 5.3 เปอร์เซนต์ ส่วนวิธี sandwichELISA วัดได้ 0, 5.3 และ 0 เปอร์เซนต์ตามลำดับ ผลการใช้ rDer f 2 และสารก่อภูมิแพ้ไรฝุ่นในการศึกษานี้ผลการตรวจแอนติบอดี IgE ด้วยวิธี sandwichELISA และ sandwichTRF ไม่แตกต่างกันทางสถิติ แต่พบว่าวิธี sandwichTRF สามารถตรวจพบแอนติบอดี IgE ได้ในระดับที่ต่ำและให้ผลที่ดีกว่าวิธี sandwichELISA ในผู้ป่วยโรคภูมิแพ้ จากผลการศึกษาสรุปได้ว่าวิธีตรวจวัดระดับแอนติบอดี IgE ด้วยวิธี sandwichTRF เป็นวิธีการทางห้องปฏิบัติการวิธีหนึ่งที่เหมาะสมในการวินิจฉัยผู้ป่วยโรคภูมิแพ้

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CHAPTER I

INTRODUCTION

House-dust mites (HDM) represent one of the most important allergen sources for the development of allergic diseases worldwide, particularly asthma and allergic rhinitis. *Dermatophagoides farinae* (Df, originally known as American HDM) and *Dermatophagoides pteronyssinus* (Dp, European HDM) are the predominant species in tropical and subtropical climates. More than 80% of allergic patients have been found to be sensitive to house dust mite allergens worldwide (73, 79). Positive skin prick tests (SPT) or serum Immunoglubulin (Ig) E antibodies to HDMs were demonstrable in allergic patients. However, if patients were allergic to histamine, SPT could lead to complicated immunoreaction harmful to the patients (122). Enzyme-linked immunosorbent assay (ELISA) was the method used to quantify serum HDM-IgE *in vitro* in laboratories (14). However, a more sensitive *in vitro* test is needed for people who had dust mite allergic clinical diagnosis but shown undetectable HDM-IgE in their sera. Monoclonal antibodies (mAbs) had been frequently used in allergy research. Their uses included the quantification of environmental allergen (18), allergens purification (35) and crystal structure (105). Specificity for unique epitopes and unlimited *in vitro* production capability are the advantage of allergen-specific mAbs.

Time-resolved fluorescent resonance energy transfer (TRF) was developed to measure Plasma vascular endothelial growth factor concentrate in 92 healthy controls, in 36 benign stomach disease patients and in 92 gastric cancer patients before surgery. (90). TRF was evaluated with a panel of serum samples from infected (n = 73) and uninfected (n = 480) sources of Brucellosis and compared to the performance of the parent cELISA, an indirect ELISA (iELISA) and fluorescence polarization assay (FPA). The performance of the TR-FRET assay matched the performance of the iELISA, which had 100% diagnostic sensitivity and specificity, and surpassed the performance of the cELISA and the FPA. The results also demonstrated that the TR-FRET technique was effective with poor-quality serum samples from the field (66).

The purpose of our study was to evaluate a rapid and convenient fluorometric method based on time resolved fluorescence of on Eu^{3+} chelate to analyze HDM-specific IgE and to compare it with the established ELISA method. We evaluated HDM-specific IgE levels in allergic patients and healthy subjects.

CHAPTER II

LITERATURE REVIEW

1. Allergic disease

Allergic diseases were produced by immunological hypersensitivity reactions to otherwise harmless substances caused by proteins called allergens. These reactions were included in the term “allergy”. The world allergy was introduced by Von Pirquet in 1906 to designate “altered reactivity” as a result of previous exposure. Allergy was mostly used as general term for reaction of discomfort of unknown origin (86). The most common allergic diseases were associated with type 1 hypersensiticity where the allergen induced IgE antibody production and sensitized T cells to produce TH2 cytokines. The effects produced by atopic or anaphylactic reaction were the results of teophase system. The sensitization phase was initiated by antigen presenting cells uptake anigen and presents the digested fragment to naïve T cells, which led to the T-cell proliferation, differentiation, cytokine production and the subsequently induced high specific IgE production from B cells. The other phase, elicitation phase, occurs upon by re-exposure to the same allergen. Following reaction, at least 2 molecules of specific IgE which bounded to the FcεRI on the mast cells and basophils were crosslinked with allergens leads to the release biological substances including histamine, heparin and serotonin. These mediators were transformed by other immunological cells into prostaglandin and leukotrienes which were responsible for the late phase allergic reaction. The effect of these mediators included smooth muscle contraction, increased vascular permeability, gastric nasal and lacrimal secretion. The type of reaction depends upon the dose of allergen, the route of contact allergen, the frequency of contact with allergen and the degree of sensitivity of involved individual. Allergic diseases were multifactorial diseases. Factors that were found to influence the occurrence of allergies were the host and environment. Host factors involved in the risk for allergy were heredity, sex, race and age, with heredity being by far the most important. Persons who had a family history of allergic diseases would be at a high risk of developing the diseases. Environmental factors could act as the trigger in agent such

as allergens, or acted like an adjuvant such as passive smoking and pollution (9). In general, an exposure dose was determined by two factors: the exposure concentration and the exposure time. For allergens, the exposure concentration was uncertain. For house dust mite, allergen particles contaminated infested fabrics and then became airborne with disturbance. Particles were cleared by settling, but some were also absorbed onto walls, furniture, and other reservoirs. Most studies of exposure had measured allergen levels in settled dust; only rarely airborne concentrations had been assessed. Settled dust and airborne dust mite allergen concentrations were highly variable, with reported coefficients of variation of 30% or more (132).

It has been reported that two reactions played a role in allergic diseases such as IgE-mediated reactions and anaphylactic reactions

1.1 IgE-mediated reactions

D. farinae and *D. pteronyssinus* were identified in house dust samples from all over the world since 1964 (121). An evidence of exposure to the HDM allergens *D. farinae* and *D. pteronyssinus* was an important risk factor for allergic sensitisation, asthma development and asthma symptom exacerbation (101). Studies of house dust-allergic individuals around the world had shown that house dust mites caused symptoms such as perennial-type asthma, rhinitis and conjunctivitis, often with nocturnal or early morning episodes (65). In Thailand, allergic conditions have been very common among children residing in Bangkok (57, 80). Teeratakulpisarn J, et al (2000) showed the four-fold rising of allergic diseases during the last decade. Number of incidences revealed that about 13% or 2,000,000 of paedetric population were suffering from allergic diseases and a large number of them were reported by the symptoms of asthma and rhinitis (118). House dust was heavily contaminated, but removal was difficult because of the inaccessible reservoirs (77).

The other constituents of native *D. pteronyssinus* allergens, but not Der p 1 or Der p 2, did not significantly influence on the IgE-mediated early asthmatic respons but contributed significantly to the allergen-induced late asthmatic response and bronchial hyperreactivity (117). *D. pteronyssinus* had also been reported to play an important role as a trigger in patients with atopic dermatitis, including adult patients (60). However, in a crosssectional study of 1669 school beginners 6 to 7 years old in Augsburg (Bavaria, Germany), it was concluded that current eczema in these children

were related to Der f 1 exposure and not to Der p 1 exposure (59). Patients who had been induced by house dust mite allergen for more than 48 hours continuously and contributed to eczematous eruptions were characterised by considerably increased levels of IgE antibodies, high activity of atopic dermatitis (50). It was possible that a number of features described to *D. pteronyssinus* would be applicable to *D. farinae* but had not specifically been investigated for this mite. The reader was referred to the entry on *D. pteronyssinus*. Various studies reported that the rate of sensitisation was higher among atopic children and that high mite infestation increased the rate of sensitisation. The European Community Respiratory Health Survey, an international study of asthma prevalence and risk factors for asthma, collected information on IgE antibodies to common allergens in over 13,000 adults living in 37 centres in 16 countries and found a median prevalence of 20.3% (range 6.7-35.1%) for sensitisation to *D. pteronyssinus* (13). In a follow up study, home visits with 3580 participants from 22 study centres in the European Community Respiratory Health Survey II were conducted; mattress dust was sampled and analysed for Der p 1 and Der f 1, which allergens were detectable (≥ 0.1 mg/g) in 68 and 53% of the samples, respectively. Large differences in allergen levels among study centres were observed, and geographic patterns for Der p 1 and Der f 1 were different. Low winter temperatures reduced Der p 1 but not Der f 1 (133). *D. farinae* and *D. pteronyssinus* appear to be significant allergens in most geographic regions but may vary within these regions. In a study in the homes of 111 asthmatic children in 3 climatic regions in Sweden, the major allergen Der m 1, together with Der p 1 from *D. pteronyssinus* and Der f 1 from *D. farinae* was analysed. Der f 1 was the predominant house dust mite allergen, Der p 1 was the least often found, and Der m 1 represented 31% of the allergen load. However, in the Linkoping area Der m 1 was the major house dust mite allergen (58%). Of the children with IgE antibodies against house dust mite, 67% reacted to all 3 mites. Mite sensitisation rates were marginally increased (7%) by the addition of IgE analysis of *D. microceras* to the routine analysis of IgE antibodies against *D. pteronyssinus* and *D. farinae*. The authors concluded that Der m 1 may in this instance be an important house dust mite allergen and should be considered when House dust mite exposure data were assessed in areas with a climate like that of Sweden (119). However, in another Scandinavian population, in Denmark, a study found that both immunochemically and microscopically, *D. farinae* was

dominant, *D. pteronyssinus* less frequent but important, and *D. microceras* insignificant (93). In a study assessing specific allergen content in dust samples from the homes of 106 allergy clinic patients in Baltimore in the USA, House dust mite allergens were detected in 99% of homes. *D. farinae* was found in 95%, *D. pteronyssinus* in 88% and *D. microceras* in 31%. Although sensitisation to these allergens was not evaluated, the study indicates that *D. microceras* may be an important allergen in this geographical region (123). In tropical Singapore, a prospective evaluation was made of 175 newly diagnosed allergic rhinitis patients, of whom 39% reported a concomitant diagnosis and/or clinical complaints of bronchial asthma and 48% of atopic dermatitis; skin reactivity for *D. pteronyssinus* and *D. farinae* mix was detected in 85% of patients (and 62% for *B. tropicalis*) (56). In studies of house dust in Bursa, Turkey, approximately 34% of houses were found to be infested with House dust mites. The rate of infestation was 18.75% and 50% in the houses with and without central heating systems, respectively. The prevalence of *D. pteronyssinus* was found to be 58.34%, compared with 16.67% for *Glycophagus domesticus* and 4.16% for *D. farinae* (41). In an evaluation of house dust collected from dwellings at 7 locations in Upper Silesia, Poland, mites were found in 56.1% of the samples. *D. farinae* was predominant (75.3%), followed by *D. pteronyssinus* (18.6%) and *Euroglyphus maynei* (1.5%) (99). A number of studies in South America had documented the significance of *D. pteronyssinus* sensitisation. In Valdivia, Chile, out of 100 consecutive paediatric asthma patients evaluated, 80 were confirmed to have skin reactivity to at least 1 mite species. All patients with skin reactivity for mites were positive to *D. pteronyssinus*, and 99% to *D. farinae*. All of the patients with severe persistent asthma had skin reactivity to mites, as did 85% in the moderate group, and 73% in the mild group. Ninety-five percent of patients with asthma and allergic rhinitis were shown to have skin reactivity to mites, as were 92% of patients with asthma and eczema and 100% of patients with asthma, allergic rhinitis and eczema (15). In a study of patients with allergic respiratory disease attending an allergy clinic in Brazil, out of 212 medical records evaluated, 61.7% showed sensitisation to Der p, 59.9% to Der f and 54.7% to Blo t (97). In a study of 579 asthmatic patients in Taiwan, it was shown through measuring allergen-specific IgE antibodies that almost 59% were sensitised to *D. microceras*, compared to 59.8% to *D. pteronyssinus* and 56.8% to *D. farinae*.

Sensitisation to Cockroach was found in 38.3%, to Dog dander in 26.3%, to *Candida albicans* in 13.3%, to Cat dander in 10% and to *Cladosporium herbarum* in 6.6%. The study indicates the importance of considering *D. microceras* when evaluating allergic individuals (22). Among 93 Taiwanese asthmatic children aged 3 to 15 years evaluated for sensitisation to 5 species of mites, 63 were found to have IgE antibodies to at least 1 of the following mites: *D. pteronyssinus*, *D. farinae*, *D. microceras*, *Euroglyphus maynei*, and *Blomia tropicalis*. Sensitisation to *D. pteronyssinus* was found in 87%, to *D. farinae* in 85%, to *D. microceras* in 84%, to *Euroglyphus maynei* in 77% and to *Blomia tropicalis* in 65% (61). Similarly, in a Taiwanese study of 498 atopic children aged 2 to 16 years, high prevalences of sensitisation were documented: 90.2% to *D. pteronyssinus,* 88.2% to *D. farinae*, 79.5% to *D. microceras*, and 76.7% to *Blomia tropicalis* (46).

A group of 25 atopic children under 11 years of age in Oxford in the United Kingdom was studied for skin reactivity and IgE antibodies to 4 species of House dust mites: *D. pteronyssinus*, *D. farinae*, *D. microceras* and *Euroglyphus maynei*. All of the children were sensitised to *D. pteronyssinus* and 80% of these children were also sensitised to *D. farinae* and *D. microceras.* Importantly, dust samples from various sites in the homes of the children revealed *D. pteronyssinus* in all the homes, but no *D. farinae* or *D. microceras*. A control group of 20 atopic children of similar ages who were not sensitised to HDM allergens had similar exposure to the 4 mite species. These results suggest that factors in addition to mite exposure were important in the development of specific IgE responses to house dust mites (130). Interestingly, in habitats were conditions were not favourable for mites, mites had still managed to survive and may cause sensitisation. The presence of *D. farinae* and *D. pteronyssinus* had been reported in Egypt (27). A large body of studies from around the world had demonstrated the relevance of this allergen (112). The reader was referred to references listed in the first paragraph of this section for more detailed clinical information.

1.2 Anaphylactic reactions

Systemic anaphylaxis could occur after the ingestion of heated or unheated mitecontaminated foods. This problem may be more prevalent in tropical and subtropical countries than previously recognised. The most common symptoms

following the ingestion of mite-contaminated flour were breathlessness, angioedema, wheezing, and rhinorrhea, and these started between 10 and 240 minutes (min) after eating (82).

2. Housedust mite allergens

As shown in Table 1, HDM allergens were grouped based on their biological function. According to the International Union of Immunological Societies (IUIS) Allergen Nomenclature Subcommittee, the designation for a characterized allergen was the first 3 letters of genus, the first letter of species name and a number the other already characterized allergen it matched in homology and molecular weight, there were 23 known mite allergens (51).

The group 1 and 2 allergens of Dermatophagoides mites were clearly major specificities. About 20% of patients, however, did not have IgE antibody to the group 1 and 2 allergens, and even though this was a minority, it constituted a large population. There were also many other house dust mite allergens which had high IgE binding activity but these were presented in low and variable concentrations in mite extracts, usually at less than 1% of the group 1 and 2 allergens (111). As previously described, the trypsin enzymatic activity of Der p 3 demonstrated a 200-fold higher concentration in spent mite media as Der f 3. (103). Studies of further relevant allergens: Der p 5, 10, 11 and 14, which appeared to be present in low quantities (40). There was evidence that Der p 3, 7 and 14 were unstable in the extracts (111); nonetheless, low amounts of allergen from crude extracts induced high titres of IgE. Nonallergenic polypeptides such as the ferritin heavy chain might be highly immunogenic and induced a balanced Th1/Th2 cytokine response (30). The following recombinant allergens had been characterised: rDer f 1 (70, 128, 11, 125, 106), rDer f 2 (70, 48), rDer f 3 (96), rDer f 6 (132), rDer f 7 (88), rDer f 11 (1, 113, 114, 63, 116), rDer f 14 (28) and rDer f 16 (107).

Table 1 Molecular cloning of *Dermatophagoides* spp. allergens.

Group	Allergen	Number of amino acid residues	Deduced MW (kDa)	Potential glycosyla-tion site(s)	Biochemical identity or homology
Group 1	Der p 1	222	24	1	Cysteine protease; homology with papain, actinidin, cathepsins B and H; sequence polymorphism
	Der f 1	223	27	1	
Group 2	Der p 2	129	15	0	NPC2 family
	Der f 2	129	15	0	
Group 3	Der p 3	232	31	0	Trypsin; homology with Der p 6 and Der f 6, mammalian and invertebrate trypsins, and other serine proteases; sequence polymorphism
	Der f 3	232	29	0	
Group 4	Der p 4	496	60	1	Amylase; homology with insect and mammalian a-amylases
Group 5	Der p 5	113	14	0	Function unknown; cleavage site 98 unknown
Group 6	Der p 6	231	25	0	Chymotrypsin; homology with Der p 3 and Der f 3, mammalian and invertebrate chymotrypsins, and other serine proteases
	Der f 6	230	25	0	
Group 7	Der p 7	198	26, 30, 31	1	Function unknown (25,30,3 1 kDa)
	Der f 7	196	30-31	1	
Group 8	Der p 8	219	27	2	GST; homology with glutathione S-transferases
Group 9	Der p 9		29		Collagenolytic serine protease
Group 10	Der p 10	284	36	0	Tropomyosin; homology with invertebrate tropomyosins
	Der f 10	284	37	0	
Group 11	Der p 11		103		Paramyosin (98 kDa); homology with invertebrate paramyosins; glycoprotein
	Der f 11		98	2	
Group 13	Der f 13				Fatty acid binding protein
Group 14	Der p 14	1650	177	0	Apolipophorin-like protein; identity with a M-l?? allergen, &E-binding Magl, Dl3961 (P39673); peptide fragments Magl and Mag3
	Der f 14		177		
Group 15	Der f 15	555	98/109	1	Chitinase (98/109 kDa, cDNA: 63 kDa)
Group 16	Der f 16	480	53	0	Gelsolin/villin, homology with gelsolin family
Group 17	Der f 17	284	53	4	EF-hand calcium-binding allergen
Group 18	Der f 18		60		Chitinase
Group 20	Der p 20				Arginine kinase
Group 21	Der p 21				
Group 22	Der f 22				
Group 23	Der p 23		14		Unknown function, homology to peritrophin-A domain (PF01607)

The major House dust mite Group 1 allergens Der p 1 and Der f 1 were the most potent of indoor allergens (106). Both allergens were major allergens and resulted in sensitisation in approximately 80% or greater of *D. farinae* sensitised patients (111). Although Der p 1 and Der f 1 were first isolated as cysteine proteases, some studies reported that natural Der f 1, similarly to Der p 1, exhibited mixed cysteine and serine protease activity (106). Der f 1 and Der f 2 sensitised approximately 80% to 90% of *D. farinae*sensitised individuals. Most patients (80%) appeared to have IgE antibodies to more than 1 allergen. Similarly to Der p 1 and Der p 2, Der f 1 and Der f 2 existed as a number of isoforms. Recombinant Der f 1 and rDer f 2 were highly homologous to the native forms. The Group 2 allergens induced humoral and cellular responses in 80-90% of mite-allergic individuals (43).

The Group 7 mite allergens reacted with IgE antibodies in 50% of sera from allergic patients (89). Der f 10, a tropomyosin, was a major allergen and had been shown to react with IgE antibodies in over 80% of mite-sensitised patients (2). Der f 11, a paramyosin, has been shown to be a major allergen. IgE reactivity to Der f 11 was reported for more than 80% of mite-sensitive asthmatic patients. Skin reactivity and IgE antibodies showed that 62% (13/21) and 50% (10/20) of mite sensitive asthmatic patients reacted positively with the recombinant Der f 11, respectively (114). This was similar to findings about rDer p 11, which detected IgE antibodies in a range of 41.7% to 66.7% in different allergic patient groups (63). In a preliminary study of 18 asthmatic children, 72.2% reacted positively to rDer f 11 and 88.9% showed positive reactivity to *D. farinae* extracts. Further evaluation of rDer f 11 in 24 asthmatic children who were skin test-positive to mite found that, whereas 70.8% had positive skin tests to rDer f 11, 75% had positive serum IgE reactivity to rDer f 11. Serum IgE reactivity to rDer f 11 was further investigated in a large panel of 49 mite skin test-positive asthmatic children, and similarly to before, 77.6% had positive serum IgE reactivity to rDer f 11 (114). Der f 14 has also been demonstrated to be a major allergen, detecting IgE antibodies in 65.8% of 38 sera samples from patients allergic to mites. Der f 14 is a proteasesensitive allergen. The breakdown products of this allergen provoked higher allergenic activity than did the intact allergen (40). HDMs cause allergic disease in dogs as well as humans. In geographical regions where the 2 mite species coexist, they both elicit specific IgE responses in humans, whereas dogs preferentially react to *D. farinae* extracts. In dogs,

the main IgE binding was directed to the *D. farinae* chitinase allergens Der f 15 and Der f 18, and not to the groups 1 and 2 allergens, as was found for humans. However, one study, aimed at characterising the chitinase allergens Der p 15 and Der p 18 of *D. pteronyssinus* and discovering whether they were important allergens for humans, as they were for dogs, reported that Der p 15 specific IgE was detected in 70% and Der p 18-specific IgE in 63% of a panel of 27 human allergic sera. The *D. pteronyssinus* chitinases Der p 15 and Der p 18 showed a high frequency of binding to IgE in allergic human sera. They were therefore potentially important allergens for humans as well as dogs (75). Recombinant Der f 16 protein was shown to bind IgE from mite-allergic patients at a 47% (8/17) frequency (52). Allergenic determinants in *D. farinae* were shared with other mites belonging to the *Pyroglyphidae* family and were highly cross-reactive with other *Dermatophagoides* species (100). There seems to be a limited cross-reactivity with Storage (nonpyroglyphid) mites (110). Allergen cross-reactivity had been reported between house dust mites and other invertebrates (92). In a study that investigated the individual allergens responsible for the cross-reactivity between *D. siboney* and other mite allergens in mite-allergic patients, IgE inhibition was shown to be higher with *D. farinae* (86%), *D. pteronyssinus* (54%) and *D. microceras* (49%) extracts than with *Lepidoglyphus destructor* (20%), *Tyrophagus putrescens* (11%), *Acarus siro* (18%) and *Blomia tropicalis* (6%) extracts. A diverse pattern for the individual allergens was demonstrated. The N-terminal sequences of Der s 1, 2 and 3 allergens showed higher homology to *D. farinae* and *D. microceras* than to *D. pteronyssinus*. The homology of the Group 2 allergens was higher than that of the Group 1 allergens. The individual allergens of *D. siboney* were more similar to those of *D. farinae* and *D. microceras* than to those of *D. pteronyssinus*. There was a limited and variable cross-reactivity with nonpyroglyphid mites. No single allergen was unique to *D. siboney* (34). Although a high prevalence of sensitisation occured to the Group 1 mite allergen Blo t 1 from *Blomia tropicalis*, there was a low correlation of IgE reactivity between this allergen and the Group 1 mite allergen Der p 1 (21) and presumably Der f 1, an allergen highly homologous to Der p 1. Pso o 1 from the Sheep scab mite (*Psoroptes ovis*) displays strong homology to the Group 1 House dust mite allergens Der p 1, Der f 1 and Eur m 1 (62). Der f 7 had a predicted 213 residue polypeptide with 86% homologies to and serological cross-reactivity with Der p 7 (88).

Der f 10 was a tropomyosin. The tropomyosin of the American cockroach *Periplaneta americana* had an 80, 81 and 82% sequence identity to the tropomyosins from *D. pteronyssinus*, *D. farinae* and Shrimp, respectively, which had been previously shown to be important allergens (83). The IgE recognition by Shrimp-allergic individuals of similar amino acid sequences, homologous to Per a 1 epitopes in mite, Cockroach and Lobster tropomyosins, indicated the basis of the *in vitro* crossreactivity among invertebrate species. On the evidence of amino acid sequence similarity and epitope reactivity, Lobster tropomyosin had been shown to had the strongest and Cockroach the weakest crossreactivity with Shrimp (6). In sera of 30 patients tested and found to harbour *P. fuliginosa*-specific IgE, the IgE binding reactivity of the *P. fuliginosa* extract was inhibited as much as 79.4% by a *B. germanica* extract and as much as 63.3% by a *D. farinae* extract. The deduced amino acid sequence of cloned cDNA was identical to that of *P. americana* tropomyosin (47). Recombinant Der p 11 showed positive IgE binding reactivity in 78% of 50 *D. pteronyssinus*sensitive asthmatic children. Der p 11, a paramyosin, had an 89% sequence identity with Der f 11 and Blo t 11 (116). A second study reported the sequence identity of Der f 11 with other known paramyosins to be 34-60% (114).

3. Immunity against House-dust mite allergen

3.1 Antibody response

There were now 23 denominated HDM allergens or IgE binding proteins. Some could drive the sensitization while others could be bystander responses. Apart from the high IgE binding of Der p 1 and 2, it was found that 3 allergens, namely Der p 4, 5 and 7 showed 10% crossreactive antibody. Generally, IgE binding to HDMs was higher than aeroallergens. Collectively, it would appear that the Der p 1, 2, 4, 5 and 7 allergens elicited 80% of the response. The IgE binding to the other allergens was low even when the prevalence was high. It had been directly demonstrated for the group 3, 8, 10 and 20 allergens (42) and because the group 6 and 9 allergens bind even less IgE than Der p 3 (126) they were low. Only occasional people had IgE to the group 13 fatty-acid-binding-protein allergens (20) and IgE binding to the group 10 tropomyosin allergen had been shown to be low in Australia (42), USA (84) and Singapore (129). High reactivity had been found in Japan (2) and Africa (120); the geographical

inconsistency suggests a cross reactivity, which was plausible because tropomyosin had a very highly conserved amino acid sequence with 70 and 80% identity to helminth and seafood allergens (32). The IgE binding for the group 16 and17 were also low in comparison to other allergens (51). IgG1 and IgG4 antibodies provided a comparison of Thl and Th2 biased responses. Many reports were confirmed early studies showing that IgG antibodies to HDM extracts were predominantly found in sera with IgE antibody (68). Greatly increased IgG1 and IgG4 had been described for Der p 1 and Der p 2 as well as pan IgG anti-Der p 1 (19). IgG1 and IgG4 levels of allergic patients and healthy subjects who had been treatment with sublingual immunotherapy did not change, while specific IgE decreased significantly in both groups (7). Sera from wheezing patients and control subjects were assayed for specific IgG, IgG4 and IgE antibody to Der p. Neither IgG nor IgG4 antibody to Der p was found. Conversely, IgE antibody was strongly associated with wheezing (31). IgG1 and IgG4 antibody titers of allergic and non-allergic children to Der p 2 were more difficult to reconcile (95). Overall the IgG1 and IgG4 antibody responses show that allergic people induced Thl and Th2 responses and both were higher than in non-allergic subjects. A comparison of IgG responses to a range of purified HDM allergens showed that both IgG1 and IgG4 antibodies were associated with the ability of the allergens to induce IgE (42).Der p 1 and 2 frequently bound both subclasses, the mid potency Der p 4, 5 and 7 bound them to a lesser degree, and the weak allergens had little activity. Studies with Western blotting also failed to reveal consistent IgG-binding components other than the major allergens (69). A caveat of the antibody binding results was other important allergens may exist. Binding of apolipophorin (group 14) and paramyosin (group 11) to IgE from sera of Japanese and Taiwanese population with high frequency and potency, respectively (40, 116). Both these allergens were labile in extracts and difficult to produce as recombinant proteins. Other potential major allergens were the group 15 and 18 chitinase allergens that were the most important allergens for dogs. The frequency of binding of recombinant chitinase polypeptides was 60-70% (75), which was high but quantitative measurements had yet to be accomplished.

3.2 T-Cell Response

T cell cloning had provided definitive evidence that non-allergic subjects make T cell responses to HDM allergens (74). Some reports had shown that PBMC

from allergicand non-allergic subjects proliferate equally well to HDM extracts or major allergens (8) but most had shown that PBMC from allergic subjects produce higher responses to HDM extracts (72). The increased proliferation had also been demonstrated for the major allergens Der p 1, Der p 2 (73), Derf 1 (16) and Der f 2 (17). An early study reported that Der p 1 could not stimulate proliferation of T cells from HDM-allergic asttnnatics, but it was inconsistent with contemporary evidence including studies from the same laboratory (91). The precursor frequencies of T cells responding to defined allergens had not been examined, but extracts had been studied. Limiting dilution assays had reported frequencies of 0.05% in mite allergic and 0.01% for non-allergic subjects (81); 0.02% in both allergic and non-allergic; 0.01% for non-allergic and 0.04-0.09% for allergic subjects (12) and 0.2 and 0.8% (53). Limiting dilution analyses of exotic antigens in naive people show frequencies of 0.001% and the frequencies found after vaccinations were about 0.02% (6). By comparison it must be concluded that the anti-HDM precursor frequencies were high even in the non-allergic people. Similar high frequencies had been found for responses to birch and grass pollen allergens (4). Anti-HDM reactive T cell clones provided some of the first evidence that Thl and Th2 cytokine polarization occurred in humans. However, it was now known that HDM allergens induced similar quantities of IFN-γ from the PBMC of allergic and non-allergic subjects. The cloning probably showed the strong in vitro polarizing activity of IL-4 produced by cells from allergic but not non-allergic subjects.

It was reported that low dose induced Thl more than Th2 cytokine; in contrast, high doses induced Th2 more than Thl cytokines. The induction of IFN-γ had been demonstrated with purified Der p 1 and Der p 2 allergens as well as extracts. Similar findings for IFN-γ release had also been found for pollen allergens, but it was possible that the IFN-γ was reduced in severe longterm allergy (64). There was little definitive data on the T cell responses elicited by purified allergens other than the major groups 1 and 2. Specific immunotherapy of Der p 1 to HDM in rhinitis and asthma patients and healtyhy subjects were observed. In healthy subjects, Der p 1 suppressed specific T cell. In patients, Der p1 suppressed proliferative T cell and Th1 (IFN-gamma) and Th2 (IL-5, IL-13) cytokine responses (49). It was showed that Der p 7 isolated from mite extracts induced similar responses to Der p 1 (88). Proliferative responses of PBMC to group 14 allergens had been shown to be similar to those

induced by major allergens by two research groups (28). The group 14 allergens had been difficult to produce or purify in the quantities required for T cell studies, so the studies had been limited to stimulation by peptides. Studies with extracts had also shown that T cell responses to antigens other than groups 1 and 2 allergens could be high (56).

4. Laboratory diagnosis of allergic diseases

Either monoclonal or polyclonal antibody has been used to measure mite allergens in the dust extract. More or less modified ELISA assays has been developed, using purified allergen or mite-allergen extracts with known, quantified amounts of allergen as standards. Monoclonal antibodies recognized just one epitope, which could be present on all isoforms of the allergen to be measured, or only on some of them; whereas polyclonal antibody recognized all epitopes/isofonns. The content of the major allergen of the mite *Dermatophagoides siboney*, Der s 1, of different batches of *D. siboney* extracts assayed with monoclonals directed against Der s 1 differed unpredictably between batches from values obtained with a kit for determination of the highly cross-reactive mite allergen Der f 1 for estimation of the content of Der s 1. Sometimes the Der f 1 kit gave higher values than the Der s 1 kit, probably because of varying isoallergen recognition. Thus, one must consider that different monoclonals did not give the same result. The data obtained were not absolute, nor universally valid. Furthermore, the method of purification of the major allergen used as standard was important. If affinity chromatography with monoclonal antibody was used to purify the major allergen used as standard, only a few isoallergens may be purified, and the standard curve would depend not on the total amount of the allergen, but on the presence of those isoforms. Therefore, the use of polyclonal antibody in these assays was an advantage (33).

4.1 In *vivo*

The skin prick test (SPT) was first described by Blackley in 1873 as a means of demonstrating pollen sensitization (108). This was a safe, practical and highly patient- and parent-acceptable way to look at allergen sensitivity in infants and children. A small amount of standardized allergen was introduced epicutaneously, using a standard single- or double-tined lancet. It was important note that it was no longer

accepted practice to use a small-gauge hypodermic needle, or to use a single lancet for multiple allergens (10). Allergen cross-bridging of IgE fixed on mast cells resulted in release of vasoactive histamine and other mediators of inflammation. Within 10 min a palpable itchy 'urticarial' papule or wheal appeared, not unlike a mosquito bite. The wheal was measured using a ruler, and recorded either as a mean of two perpendicular diameters (recorded in mm) or as an area, using a computerlinked laser reader. The older practice of comparing the wheal size to that of response to the histamine control was no longer supported, as clinicians could now use absolute wheal size expressed in mm to predict clinical reactivity, as assessed in a food challenge (104). However, it was still important to always use a histamine positive control, to ensure that the child was actually able to mount a wheal and flare response (thereby validating any wheal response that was elicited by an allergen) and was not taking an anti-histamine, which would block such a response. A negative (saline) control was always used to ensure that the child did not have dermographism or pressure sensitivity. The SPT was undertaken in infants and young children and noted for its safety and acceptability. It could be performed in low-risk children in home or school settings (102). Most adverse reactions to SPT occur in subjects with unstable allergic conditions, particularly inhalant allergen-associated asthma. These children were identifiable by history and should had SPT performed in a hospital setting (24). In older children and adults the volar (palmar) aspect of the forearm(s) was used; in infants the back was the best, as the infant could be held and comforted against a parent or assistant. Allergen SPT was difficult if there was widespread eczema. It was said to be unreliable soon after an anaphylactic episode (when the mast cells had all degranulated and require "re-stocking" (87). Apart from anti-histamines, common drugs had little effect and oral steroids and topical calicineurin inhibitors for eczema such as tacrolimus appear to have no adverse effect.

4.2 In *vitro*

It had been reported that specific IgE and IgG subclass antibodies against whole body antigen and two major allergens of *Dermatophagoides farinae* (Der f 1 and Der f 2) were measured in sera from 66 adults with asthma and 34 normal subjects by ELISA. The mean optical density (OD) values of specific IgE and IgG subclass antibodies in 100 studied sera were significantly higher than those of the two major allergens ($p<0.001$) and the level of Der f 2- IgG1, IgG4 and IgE were higher

than those of Der f 2 but IgG2 of Der f 1 was higher than that of Der f 2 ($p<0.001$). Among IgG subclass antibodies of Der f 1, IgG2 was significantly higher in the nonatopic group than in the atopic group. Among IgG subclass antibodies of Der f 2, IgG2 and IgG4 were significantly higher in the atopic group than in the nonatopic group (45).

The presence of IgE, IgG1 and IgG4 to concanavalin A-binding antigens (Bt-Con-A) isolated from *Blomia tropicalis* (Bt)-total extract in sera of allergic and non-allergic subjects were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and ELISA in sera of 121 patients with allergic rhinitis and 36 non-allergic individuals. All subjects were skin prick tested with Bt-total extract and inhibition tests were performed for IgE, IgG1 and IgG4 using both extracts (Bt-total and Bt-Con-A). Skin prick test showed that 58% of the patients were sensitized to Bt (Bt+), with 52% reactive to both mites (Bt and Dp) and 6% to Bt only. ELISA showed a similar profile of IgE, IgG1 and IgG4 levels in response to Bt-total and Bt-Con-A extracts in different groups, although Bt+ patients showed a lower IgG4 reactivity to Bt-Con-A extract. Specific IgG1 levels were higher in Bt+ patients than in control subjects, and IgG4 levels showed no significant difference among groups. ELISA inhibition showed a partial IgE and total IgG1 and IgG4 cross-reactivity with *D. pteronyssinus* extract for Bt-total and Bt-Con-A extracts (3). From previous study, it was shown that ELISA was rapid, simple and specific technique for determination of specific IgE to house dust mite, although it was less sensitive than skin prick test. However, development of more sensitive assays was essential to enable the quantification of the minute amount of specific allergen present in the crude extract and the environment.

After europium (Eu) chelates were developed as labels in the 1980s (94), TR-IFMA had been reported as an ideal immunoassay technique. Time-resolved immunofluorometric assays (TR-IFMA) had been reported as an ideal immunoassay technique. Sensitivity was higher with this technique than with methods based on tritiated tracers or enzyme-conjugated tracers. In addition, TR-IFMA offers an alternative, challenging immunoassays based on 125I labels with the advantage of a stable, non-radioactive tracer (37). Fluorescence of the Eu3+ tracer was long-lived and allows for the differentiation of the short-lived background fluorescences of biological

material, plastics and optics (98). The highly specific activity of the label increases the sensitivity of immunoassays while minimizing non-specific binding of the labelled bioaffinity molecule. Other favourable features of Eu3+ complexes had been reviewed (25, 26, 55). Recently, TR-IFMA was developed to measure Plasma vascular endothelial growth factor (VEGF) concentrate in 92 healthy controls, in 36 benign stomach disease patients and in 92 gastric cancer patients before surgery. A standard curve for VEGF TR-IFMA had been developed with good sensitivity (0.37 pg/ml). Accuracy studies, specificity, parallelism and precision data were determined and all were found to be satisfactory. The validity of the VEGF assay was confirmed by the good correlation between the results obtained by TR-IFMA and ELISA (ELISA result = 1.862 + 0.953 (TR-IFMA result), r = 0.944. At the cut-off of 217.79 pg/ml, the diagnostic sensitivity, specificity and accuracy of the TR-IFMA were 40.2, 93.7 and 69.9%, respectively (90). Moreover, time-resolved fluorescent resonance energy transfer (TR-FRET) assay was evaluated with a panel of serum samples from infected (n = 73) and uninfected (n = 480) sources of Brucellosis and compared to the performance of the parent cELISA, an indirect ELISA (iELISA) and fluorescence polarization assay (FPA). The performance of the TR-FRET assay matched the performance of the iELISA, which had 100% diagnostic sensitivity and specificity, and surpassed the performance of the cELISA and the FPA. The results also demonstrated that the TR-FRET technique was effective with poor-quality serum samples from the field (66). In addition to TR-FRET, double-antigen sandwich time-resolved immunofluorometric assay (DAS-TR-IFMA) had been developed to detect total anti-HCV antibodies based on biotin-streptavidin interaction. For comparison, 1025 samples were analysed by the DAS-TR-IFMA and three indirect anti-HCV antibody detection methods. For samples with discordant results, PCR-ELISA and Inno-LIA were employed as supplementary assays to analyse the presence of HCV antibodies. With regard to the 1025 clinical samples, the overall concordance between the DAS-TRIFMA and the three indirect methods was 99.41, 98.93 and 98.93 % for Ortho ELISA 3.0, WAT ELISA and I-TR-IFMA, respectively. The specificity/sensitivity of the DAS-TRIFMA, Ortho HCV ELISA 3.0, WAT HCV ELISA and I-TR-IFMA were 100%/99.09%, 99.34%/98.18%, 99.23%/97.27% and 99.01%/98.18%, respectively. The DAS-TR-IFMA was able to detect HCV antibodies at a concentration about 1/10

of that detectable by Ortho HCV ELISA 3.0, WAT HCV ELISA and I-TR-IFMA. It was concluded that the DAS-TR-IFMA was a more specific and reliable method for screening anti-HCV antibodies (124).

The purpose of our study was to evaluate a rapid and convenient fluorometric method based on time resolved fluorescence of on Eu^{3+} chelate to analyze HDM-specific IgE and to compare it with the established ELISA method. We evaluated HDM-specific IgE levels in allergic patients and healthy subjects.

CHAPTER III

MATERIALS AND METHODS

1. House-dust mite (HDM) allergens

Total HDM extracts of *D. farinae* (Df) and *D. pteronyssinus* (Dp) were obtained from mite cultures provided by Siriraj Dust Mite Center of Services and research, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

1.1 Preparation of House dust mite allergen

Briefly, mite culture was stirred in phosphate buffered saline (PBS), pH 7.4 for overnight at 4 degree Celsius (°C). The mixture was centrifuged for 20 min at 12,000xg at 4°C. The supernatant was filtered through No.1 Whatman paper and through 0.22 μ filter. The sterile mite extract was then stored frozen at -20°C (18).

Recombinant allergen group 2 of *Dermatophagoides farinae* (rDer f 2) was produced by donated from Assistant Professor Surapon Piboonpocanun from the Institute of Molecular Biosciences, Mahidol University, Thailand.

The positive transformants containing the integration of interested genes were selected for small-scaled expression, Briefly, positive transformants (rDer f 2) and plasmic vecter (pPICZ) of *Pichia pastoris* (*P. pastoris*) were grown in 2 milliliters (ml) of yeast extract peptone dextrose (YPD) containing 100 μg/ml Zeocin™ at 30 °C with vigorous shaking (250 rpm) for approximately 2 days. The starter cultures were added into 5 ml of fresh buffered minimal glycerol complex medium (BMGY) and incubated at 30 °C until the absorbance of BMGY was 0.1 at optical density (OD) 600 nanometer (nm). After that, the starter cluture with OD 0.1 were cultured and harvested until the absorbanes at of BMGY were 5.0. The BMGY cultures were centrifuged at 10,000xg for 5 min, and the cell pellets were resuspended in buffered minimal methanol complex medium (BMMY) using 1/5 volume of the original culture volume (approximately 1 ml). To induce expression of recombinant allergens, absolute methanol was added to a final concentration of 3% v/v to the BMMY culture every 24 h. The culture media were collected after 3 day induction. The collected culture media were centrifuged

at 12,000xg in a bench-top microcentrifuge for 3 min at room temperature. The supernatant was transferred to a separate tube and were stored frozen at -20 °C until used.

1.2 Determination of HDM protein

Protein concentration of HDM was determined by using the BioRad Protein Assay (dye-binding assay), (BioRad, Laboratory, CA) based on the method of Bradford. For standard protein, bovine serum albumine (Sigma, Steinheim, Germany) concentration was ranged from 0.0625 to 1 mg/ml was used. Ten microliters of each HDM protein was mixed with 90 µl of acid Comassie blue dye and incubated at room temperature. After incubation for 5 min, the mixture was measured for the OD 595 nm.

1.3 Characterization of HDM allergen

Characterize the protein components of the extracted antigen were prepared from *D. farinae* and *D. pteronyssinus* by using SDS-PAGE. The SDS-PAGE was using 4% stacking gel and 12.5 % resolving gel. The extracted protein had been reduced and denatured by mixing with 4X sample buffer and then boiled in a water bath at 95°C for 10 min before loading onto the gel. The protein content used to load the gel was approximately 2 µg per lane. Electrophoresis was carried out with a constant voltage of 100 volts (v) until the front dye reaches the bottom of the gel. After that, the gel was stained with Coomassie Brilliant Blue.

Proteins were firstly separated by SDS-PAGE followed by transferring the fractionated proteins to a nitrocellulose membrane as prescribed by the manufacturer with a wet blotting apparatus (Bio-Rad, USA). The blotted membrane was blocked to prevent non-specific binding of antiserum to the membrane by gently rocking overnight at 4 °C in blocking solution. The blot was then washed by shaking vigorously in washing buffer for 5 min. The washing step was performed in six times replica. The blot was then incubated with continuous shaking at room temperature for 120 min in a 1:2000 dilution of monoclonal anti-group 2 antibodies (mAb 1D8 clone, Indoor biotecnologies Ltd, Manchester, UK). After six-time washing, the membrane was incubated with biotinylated rabbit-anti mouse IgG solution (1:5000) (SouthernBiotech, Birmingham, USA) for another 60 min at room temperature. After

six-time washing, the membrane was incubated with streptavidin-AP solution (1: 2000) (KPL, USA) for another 30 min at room temperature. The membrane was then washed with the same condition and the band of specific proteins was visualized by adding BCIP/NBT 1 component substrate (KPL, USA) and incubating at room temperature with gently shaking until color was developed.

2. Subjects

Fifty rhinitis patients with or without intermittent or persistent, mild-to-moderate asthma with positive SPT were selected for this investigation by Dr. Nares Wongpitoon and Dr. Voravan Durongpisitkul of Department of Pediatric, Police General Hospital, and Bangkok, Thailand during October 2008 to October 2009. For the control group, 19 healthy subjects from Silpakorn University 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. The allergen extract of commercial Der p (*Dermatophagoides pteronyssinus*), Der f (*D. farinae*), CR (American Cockroach), Grass (Bermuda grass pollen), Dog (Dog Epithelium), Cat (Cat pelt), Wheat, Shrimp, Feather (Chicken, Duck, Goose), M1 (*Penicillium notatum*, *Aspergillus niger*, Drechslera, *Cladosprium spharospermum*, Alternaria), M2 (Curvularia, Fusarium, Aureobasidium, Mucor, Rhizopus) were used to screen allergic patients, accordind to the manufacture's instructions. Histamine dihydrochloride (10 mg/ml) was used as positive control and normal saline (0.9% w/v) was used as negative control.

SPT was performed on the skin of volunteer forearm using allergen extracts as mentioned above along with positive and negative controls. Briefly, the arm was first cleaned, then a drop of Allergen extract or control solution was placed onto a marked area of skin, with a distance of 2-3 centimeter (cm) apart. A sterile No 23G needle was used to prick the skin through the allergen drop, which allowed a small amount of allergen enter the skin. Each allergen was prickd with a separate needle. Two to three minutes later, the residue fluid on the skin was carefully wiped off to avoid mixing the adjacent samples. The reaction was measured after 15 min by copying the wheal size with transparent adhesive and kept tape onto a record sheet for later analysis. All the

patients were observed for another had for any unpleasant reactios such as local itch and swelling, local rash, generalized rach or anaphylaxis.

3. Immunological analysis

Ten ml of venous blood was received from each individual. Blood was centrifuged at 400g for 30 min., RT. The serum was then collected and frozen at -20 °C until used

3.1 Enzyme-linked immuosorbent assay (ELISA)

IgE reactivity to a panal of proteins from Df, Dp, rDer f 2 and pPICZ was determined by ELISA. Briefly, dilute each allergen in coating buffer (50 nM carbonate/bicarbonate buffer) at concentration of 1000 ng/ml. One-hundred µl of each allergen was coated in each well of 96-well poly polystyrene microtiter wells (Nunc™, Denmark) and incubated at 4 °C overnight. The blocking buffer was added into each well and incubated at 37 °C for 60 min. The sera at dilution of 1: 10 was added into the well and incubated at 37 °C for 120 min. Biotinylated-labeled mouse anti-human IgE antibody (SouthernBiotech, Birmingham, USA) at dilution of 1: 1000 was added into each well and incubated at 37 °C for 60 min. The plates was added with streptavidin-peroxidase solution at dilution of 1: 1000 and incubated at 37 °C for 60 min. During each step, the plates were washed with PBS-Tween20 for 5 times. The color reaction was developed by adding ABTS® peroxidase substrate (KPL, USA). Absorbance of IgE antibody was measurced at OD405nm with a Wallac 1420 microplate reader (Auto DELFIA Wallac 1420; Wallac, Turku, Finland).

For assay optimization. There were many parameters which influence the result in ELISA. These include antigen, antibody and conjugate concentration. Therefore, the following conditions were evaluated.

3.1.1 Evaluation of coating concentration of allergens. Different coating concentrations of allergens in coating buffer were evaluated. The protein concentrations of Df, rDer f 2 and pPICZ vary from 2.5, 5 and 10 µg/ml.

3.1.2 Evaluation of subject's serum. One hundred microliters per well of allergen dilute to the 10 µg/ml in coating buffer were transferred to an ELISA plate. Concentrations of antigens in dilution buffer were evaluated. The protein concentrations of Df, rDer f 2, Dp and pPICZ vary from 2.5, 5 and 10 µg/ml.

3.1.3 Evaluation of biotinylated-labeled mouse anti-human IgE antibody. One hundred microliters per well of allergen dilute to the 10 µg/ml in coating buffer were transferred to an ELISA plate. Concentrations of antigens in dilution buffer were evaluated. The concentrations of biotinylated-labeled mouse anti-human IgE antibody vary from 1: 1000 and 1: 2000.

3.2 Time resolved fluoroimmunoassay (TRF)

IgE reactivity to a panal of proteins from Df, Dp, rDer f2 and pPICZ was determined by TRF. Briefly, dilute each allergen in coating buffer (50 nM carbonate/bicarbonate buffer) at concentration of 1000 ng/ml One-hundred µl of each allergen was coated in each well of 96-well poly polystyrene microtiter wells (Nunc™, Denmark) and incubated at 4 °C overnight. The DELFIA® Assay Buffer was added into each well and incubated at 37 °C for 60 min. The sera at dilution of 1: 10 in DELFIA® Assay Buffer was added into the well and incubated at 37 °C for 120 min. Biotinylated-labeled mouse anti-human IgE antibody (SouthernBiotech, Birmingham, USA) at dilution of 1: 1000 was added into each well and incubated at 37 °C for 60 min. The plates was added with DELFIA® EU-labeled Streptavidin at dilution of 1: 1000 and incubated at 37 °C for 60 min. During each step, the plates were washed with DELFIA® Wash concentrate for 5 times. The color reaction was developed by adding DELFIA® Enhancement Solution. Absorbance of IgE antibody was measurced at OD615nm with a Wallac 1420 microplate reader (Auto DELFIA Wallac 1420; Wallac, Turku, Finland).

3.3 Sandwich enzyme-linked immuosorbent assay (sandwichELISA)

IgE reactivity to a panal of proteins from Df, Dp, rDer f 2 and pPICZ was determined by sandwichELISA. Briefly, dilute monoclonal anti-group 2 antibodies dilution of 1:1000 in coating buffer (50 nM carbonate/bicarbonate buffer) add 100 µl was coated in each well of 96-well poly polystyrene microtiter wells (Nunc™, Denmark) and incubated at 4 °C overnight. The allergens at concentration of 1000 ng/ml was added into the well and incubated at 37 °C for 120 min. The sera at dilution of 1: 10 was added into the well and incubated at 37 °C for 120 min. Biotinylated-labeled mouse anti-human IgE antibody (SouthernBiotech, Birmingham, USA) at dilution of 1: 1000 was added into each well and incubated at 37 °C for 60 min.

The plates was added with streptavidin-peroxidase solution at dilution of 1: 1000 and incubated at 37 °C for 60 min. During each step, the plates were washed with PBS-Tween20 for 5 times. The color reaction was developed by adding ABTS® peroxidase substrate (KPL, USA). Absorbance of IgE antibody was measurced at OD405nm with a Wallac 1420 microplate reader (Auto DELFIA Wallac 1420; Wallac, Turku, Finland).

3.4 Sandwich time resolved fluoroimmunoassay (sandwichTRF)

IgE reactivity to a panal of proteins from Df, Dp, rDer f 2 and pPICZ was determined by sandwichTRF. Briefly, dilute monoclonal anti-group 2 antibodies dilution of 1:1000 in coating buffer (50 nM carbonate/bicarbonate buffer) add 100 µl was coated in each well of 96-well poly polystyrene microtiter wells (Nunc™, Denmark) and incubated at 4 °C overnight. The allergens at concentration of 1000 ng/ml was added into the well and incubated at 37 °C for 120 min. The sera at dilution of 1: 10 in DELFIA® Assay Buffer was added into the well and incubated at 37 °C for 120 min. Biotinylated-labeled mouse anti-human IgE antibody (SouthernBiotech, Birmingham, USA) at dilution of 1: 1000 was added into each well and incubated at 37 °C for 60 min. The plates was added with DELFIA® EU-labeled Streptavidin at dilution of 1: 1000 and incubated at 37 °C for 60 min. During each step, the plates were washed with DELFIA® Wash concentrate for 5 times. The color reaction was developed by adding DELFIA® Enhancement Solution. Absorbance of IgE antibody was measurced at OD615nm with a Wallac 1420 microplate reader (Auto DELFIA Wallac 1420; Wallac, Turku, Finland).

In order to quantify specific IgE levels, serum from three highest HDM-sensitized individuals as analyzed by sandwich ELISA and sandwich TRF were analyzed by the ImmunoCAP™ system (Pharmacia Diagnostic AB, Uppsala, Sweden) 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 kilo units of aprotinin-specific antibodies per liter (kU_A/l) of Df, rDer f 2 and Dp specific IgE respectively were made for use in calibrating curve of both sandwich ELISA and sandwich TRF.

4. Statistic analysis

Levels of specific IgE to HDM allergens in sandwich ELISA and sandwich TRF were obtained from linear regression equation. All statistic calculations were performed using SPSS Version 13 (Chicago, IL, USA).

CHAPTER IV

RESULTS

1. HDM–allergen characteristics

In the present study, HDM allergens consisted of native (Dp and Df) and recombinant (rDer f 2) allergens expressed in *P. pastoris*. For negative control, protein of pPICZ was used. The characters of HDM allergens were determind by SDS-PAGE and western blot, as shown in Figure 1. The molecular weight of native allergens (Df and Dp) were ranged from 10 to 72 kDa whereas that of rDer f 2 was 14 kDa. Molecular weight of pPICZ was not found. Additionally, molecular weight of rDer f 2 comfirmed by western blot was 14 kDa.

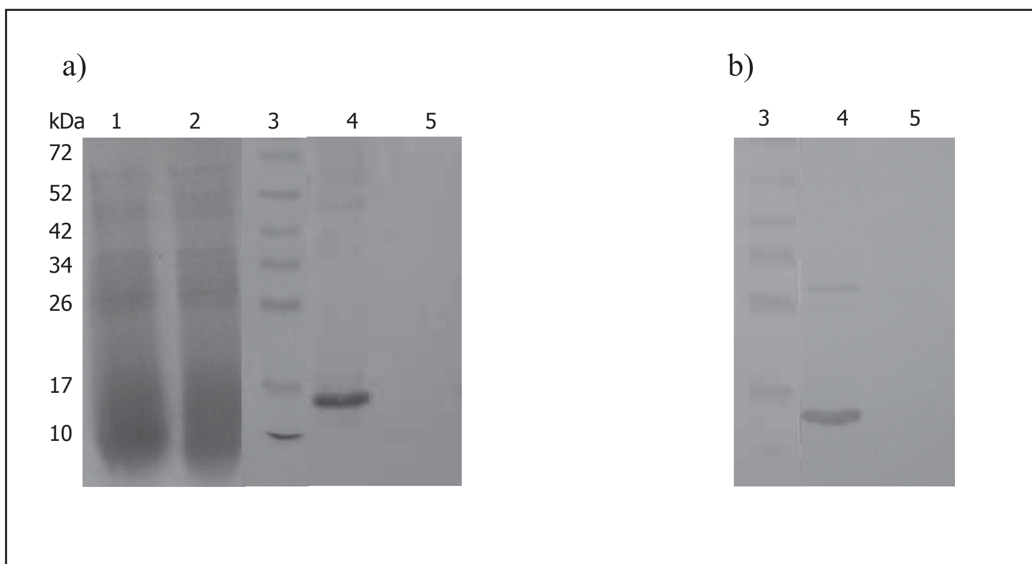

Figure 1 The protein components of HDM allergens were determined by SDS-PAGE a); Lane 1:Df; Lane 2: Dp; Lane 3: Molecular mass standards (in kDa); Lane 4: rDer f 2; Lane 5: pPICZ. b) Western blot Lane 3: Molecular mass standards (in kDa); Lane 4: rDer f 2; Lane 5: pPICZ

2. Subject characteristics

Specific antibodies to HDM allergens were examined in a total of 69 subjects who resided in Bangkok, Thailand (80). Subjects were screened by SPT using commercial allergens (Allertech, ALK, UK) as shown in Table 2. Histamine dihydrochloride (10 mg/ml) was used as positive control and normal saline (0.9% w/v) was used as negative control. As shown in Table 2, subjects could be divided into 2 groups; 50 allergic patients and 19 healthy subjects. The demographic clinical characteristics of these subjects were shown in Table 3 and 4.

Table 2 List of commercial allergens used for select the patient groups.

No.	Allergens	Concentrations
1.	Der f. (*Dermatophagoides farinae*)	10,000 AU/ml
2.	Der p. (*Dermatophagoides pteronyssinus*)	10,000 AU/ml
3.	CR (American Cockroach)	1:20 W/V
4.	Grass (Bermuda grass pollen)	10,000 BAU/ml
5.	Dog (Dog Epithelium)	15,000 PNU/ml 1:10 W/V
6.	Cat (Cat Pelt)	10,000 BAU/ml
7.	Wheat	1:10 W/V
8.	Shrimp	1:10 W/V
9.	Feather (Chicken, Duck, Goose)	1:20 W/V
10.	M1 { *Penicillium notatum* *Aspergillus niger* *Drechslera* *Cladosprium sphaerospermum* *Alternaria*	20,000 PNU/ml 1:10 W/V
11.	M2 { *Curruria* *Fusarium* *Aureobasidium* *Mucor* *Rhizopus*	20,000 PNU/ml 1:10 W/V

Table 3 Demographic and characteristics of the study individuals.

Characteristics	Groups	
	allergic individuals	healthy individuals
Number of subjects (n)	50	19
Age (years, mean ± SD)	39.52 ± 9.93	25.21 ± 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 %	

HDM, House dust mite; Der f, *D. farinae*; Der p, *Dermatophagoides pteronyssinus*; CR, American Cockroach; Grass,Bermuda grass pollen; Dog, Dog Epithelium; Cat, Cat pelt; Wheat; Shrimp; Feather, Chicken, Duck, Goose; M1, *Penicillium notatum*, *Aspergillus niger*, Drechslera, *Cladosprium spharospermum*, Alternaria; M2, Curvularia, Fusarium, Aureobasidium, Mucor, Rhizopus

Table 4 Profiles of 69 subjects responsed to panel of allergens sensitization. (A= allergic individuals, H= healthy subjects, M=male, F=Female, -=not determine)

			Antigens sensitization (wheal ≥ 3 mm)											
ID	**Sex**	**Age**	**Der f**	**Der p**	**CR**	**Grass**	**Dog**	**Cat**	**Wheat**	**shrimp**	**Feather**	**M1**	**M2**	**etc**
A1	F	29	+	+	+	-	-	-	-	-	-	+	+	-
A2	M	25	+	+	-	-	-	-	-	-	-	-	-	-
A3	F	45	+	+	-	-	-	-	-	-	-	-	-	-
A4	M	36	+	+	-	-	-	-	-	-	-	-	-	-
A5	F	40	+	+	+	-	-	-	-	-	-	-	-	-
A6	F	34	+	+	-	-	-	-	-	-	-	-	-	-
A7	M	28	+	+	-	-	-	-	-	-	-	-	-	-
A8	F	40	+	+	-	-	-	-	-	-	-	-	-	-
A9	M	48	+	+	-	-	-	-	-	-	-	-	-	+
A10	F	28	+	+	-	-	-	-	-	-	-	-	-	-
A11	F	43	+	+	+	+	-	-	-	-	-	-	-	-
A12	M	52	+	+	-	-	-	-	-	-	-	-	-	-
A13	M	49	+	+	+	-	-	-	-	-	-	-	-	-
A14	F	41	+	+	-	-	-	-	-	-	-	-	-	-
A15	F	41	+	+	-	-	-	-	-	-	-	-	-	-
A16	F	42	+	+	-	-	-	-	-	-	-	-	-	-
A17	M	43	+	+	-	-	-	-	-	-	-	-	-	-
A18	M	56	+	+	-	-	-	-	-	-	-	-	-	-
A19	M	18	+	+	-	-	-	-	-	-	-	-	-	-
A20	M	47	+	+	-	-	-	-	-	-	-	-	-	-
A21	F	45	+	+	-	-	-	-	-	-	-	-	-	-
A22	F	41	+	+	-	-	-	-	-	-	-	-	-	-
A23	M	25	+	+	-	-	-	-	-	-	-	-	-	-
A24	M	28	+	+	-	-	-	-	-	-	-	-	-	-
A25	M	43	+	+	-	-	-	-	-	-	-	-	-	-
A26	M	40	+	+	+	-	+	-	-	-	-	-	-	-
A27	M	22	+	+	-	-	-	-	-	-	-	+	-	-
A28	F	49	+	+	-	-	-	-	-	-	-	-	-	-
A29	F	40	+	+	-	-	-	-	-	-	-	+	-	-
A30	F	40	+	+	+	+	-	-	-	-	-	-	-	-
A31	M	53	+	+	-	-	-	-	-	-	-	-	-	-
A32	F	55	+	+	+	+	-	-	-	-	-	-	-	-
A33	F	29	+	+	-	-	-	-	-	-	-	-	-	-
A34	F	49	+	+	+	-	-	-	-	-	-	-	-	-
A35	M	51	+	+	-	-	-	-	-	-	-	-	-	-

Table 4 (continue) Profiles of 69 subjects responsed to panel of allergens sensitization. (A= allergic individuals, H= healthy subjects, M=male, F=Female, -=not determine)

			Antigens sensitization (wheal ≥ 3 mm)											
ID	**Sex**	**Age**	**Der f**	**Der p**	**CR**	**Grass**	**Dog**	**Cat**	**Wheat**	**shrimp**	**Feather**	**M1**	**M2**	**etc**
A36	F	52	+	+	-	-	-	-	-	-	-	-	+	-
A37	F	49	+	+	-	-	-	+	-	-	-	+	-	-
A38	M	43	+	+	-	-	-	-	-	-	-	-	-	-
A39	M	46	+	+	-	-	-	-	-	-	-	-	-	-
A40	F	49	+	+	-	-	-	-	-	-	-	-	-	-
A41	M	46	+	+	+	-	-	+	-	-	-	+	-	-
A42	M	23	+	+	-	-	-	+	-	-	-	-	-	-
A43	M	35	+	+	-	-	-	+	-	-	-	-	-	-
A44	M	47	+	+	-	-	-	-	-	-	-	-	-	-
A45	F	30	+	+	-	-	-	-	-	-	-	-	-	-
A46	F	39	+	+	-	+	+	-	-	-	-	-	-	-
A47	F	39	+	+	-	-	-	-	-	-	-	+	+	-
A48	M	39	+	+	+	-	-	-	-	-	-	-	-	-
A49	F	22	+	+	-	-	-	-	-	-	-	-	-	-
A50	F	22	+	+	-	-	-	-	-	-	-	-	-	-
H1	F	32	-	-	-	-	-	-	-	-	-	-	-	-
H2	F	25	-	-	-	-	-	-	-	-	-	-	-	-
H3	F	27	-	-	-	-	-	-	-	-	-	-	-	-
H4	F	25	-	-	-	-	-	-	-	-	-	-	-	-
H5	F	32	-	-	-	-	-	-	-	-	-	-	-	-
H6	F	27	-	-	-	-	-	-	-	-	-	-	-	-
H7	F	48	-	-	-	-	-	-	-	-	-	-	-	-
H8	M	25	-	-	-	-	-	-	-	-	-	-	-	-
H9	F	24	-	-	-	-	-	-	-	-	-	-	-	-
H10	M	20	-	-	-	-	-	-	-	-	-	-	-	-
H11	F	22	-	-	-	-	-	-	-	-	-	-	-	-
H12	F	21	-	-	-	-	-	-	-	-	-	-	-	-
H13	M	19	-	-	-	-	-	-	-	-	-	-	-	-
H14	M	20	-	-	-	-	-	-	-	-	-	-	-	-
H15	F	19	-	-	-	-	-	-	-	-	-	-	-	-
H16	F	20	-	-	-	-	-	-	-	-	-	-	-	-
H17	F	22	-	-	-	-	-	-	-	-	-	-	-	-
H18	F	21	-	-	-	-	-	-	-	-	-	-	-	-
H19	F	30	-	-	-	-	-	-	-	-	-	-	-	-

3. The optimization of ELISA condition for specific IgE detection

The pooled positive sera from allergic patient and negative sera from healthy subject were used as a positive and negative control for ELISA optimization.

3.1 The optimization of allergen concentration

Various concentrations of Df, rDer f 2 and pPICZ (250, 500, 1000 ng/ml) were used for coating the plate. As shown in Table 5, the allergic patient specific IgE detected with Df, rDer f 2 and pPICZ were 0.133, 0.490 and 0.277 unit, respectively at OD405nm. The healthy subject specific IgE detected with Df, rDer f 2 and pPICZ were 0.133, 0.293 and 0.260 unit, respectively at OD405 nm. These results indicated that the optimal concentrations of all allergens including pPICZ were 1000 ng/ml.

3.2 The optimization of serum dilution

Various dilutions of sera (1:2 and 1:10) were used for detection of specific IgE. As shown in Table 6, the allergic patient specific IgE detected with Df, rDer f 2, Dp and pPICZ were 0.185, 0.331, 0.137 and 0.144, respectively at OD405 nm. The healthy subject specific IgE detected with Df, rDer f 2, Dp and pPICZ were 0.125, 0.225, 0.122 and 0.115 unit, respectively at OD405 nm. The result indicated that the optimal dilutions for further work of sera were 1:10.

3.3 The optimization of biotinylated-labeled mouse anti-human IgE antibody

Various dilutions of biotinylated-labeled mouse anti-human IgE antibody (1:1000 and 1:2000) were used for detection of soecific IgE. As shown in Table 7, the allergic patient specific IgE detected with Df, rDer f 2 and pPICZ were 0.133, 0.495 and 0.277 unit, respectively, at OD405 nm. The healthy subject specific IgE detected with Df, rDer f 2 and pPICZ were 0.133, 0.350 and 0.260 unit, respectively at OD405 nm. The result revealed that the optimal dilutions of biotinylated-labeled mouse anti-human IgE antibody for further work were 1:1000.

However, the highest concentrations of antigen detected in positive sera similar to negative sera. This indicated that coating of the well using allergen was not suitable to separate specific IgE of HDM-specific subject and healthy subject specific IgE, Sandwich ELISA using mAb was then recommened for further use.

Table 5 Optimization of Df, rDer f 2 anf pPICZ concentrations for the detection of specific IgE. Various allergens concentrations (250, 500 and 1000 ng/ml) were evaluluated in the experiments. The serum and biotinylated- labeled mouse anti-human IgE antibody diluted 1:10 and 1:1000, respectively by ELISA.

Subjects	Allergens concentrations (ng/ml)								
	Df			rDer f 2			pPICZ		
	250	500	1000	250	500	1000	250	500	1000
Allergic patient	0.119	0.115	0.133	0.351	0.359	0.490	0.285	0.280	0.277
Healthy subject	0.120	0.114	0.133	0.240	0.293	0.35	0.288	0.279	0.260

Table 6 Optimization of Df, rDer f 2, Dp and pPICZ concentrations for the detection of specific IgE. Allergens concentrations 1000 ng/ml was evaluluated in the experiments. The biotinylated-labeled mouse anti-human IgE antibody diluted 1:1000. The serum diluted 1:2 and 1:10, respectively by ELISA.

Allergens	Serum dilution			
	Allergic patient		Healthy subject	
	1:2	1:10	1:2	1:10
Df	0.159	0.185	0.140	0.125
rDer f 2	0.199	0.331	0.240	0.255
Dp	0.148	0.137	0.123	0.122
pPICZ	0.096	0.144	0.140	0.115

Table 7 Optimization of Df, rDer f 2 and pPICZ concentrations for the detection of specific IgE. Allergens concentrations 1000 ng/ml was evaluluated in the experiments. The serum diluted 1:10. The biotinylated- labeled mouse anti-human IgE antibody diluted 1:1000 and 1:2000, respectively by ELISA.

Allergens	Biotinylated-labeled mouse anti-human IgE antibody dilution			
	Allergic patient		Healthy subject	
	1:1000	1:2000	1:1000	1:2000
Df	0.133	0.108	0.133	0.144
rDer f 2	0.495	0.408	0.350	0.335
pPICZ	0.277	0.262	0.260	0.274

4. Calibration curve of specific IgE detected with all HDM allergens

4.1 Calibration curve between OD unit and kU_A/l units sandwichELISA

The calibrated line of two unit method were studied in an allergic patient who had specific IgE at 34.9 kU_A/l for Df and rDer f 2 and 45.5 kU_A/l for Dp compared to a healthy subject who had specific IgE at ≤ 0.35 kU_A/l for all HDM allergens. The solid line represented the value of OD unit and kU_A/l, as shown in Figure 2. In allergic pateint, the highest specific IgE levels detected with Df, rDer f 2 and Dp were 3.49 kU_A/l compared with 0.547 OD unit, 3.49 kU_A/l compared with 0.568 OD unit and 4.55 kU_A/l compared with 1.301 OD unit, respectively. The lowest specific IgE levels detected with Df, rDer f 2 and Dp were 0.22 kU_A/l compared with 0.087 OD unit, 0.22 kU_A/l compared with 0.085 OD unit and 0.28 kU_A/l compared with 0.169 OD unit, respectively. In a healthy subject, the highest specific IgE levels after detection with all HDM allergens was less than 0.35 kU_A/l compared with 0.085 OD unit. The equation of specific IgE levels in both subjects between OD unit and kU_A/l unit was 10(0.098+0.1337OD unit) for Df, 10(0.0921+0.1403OD unit) for rDer f 2 and 10(0.1579+0.2613OD unit) for Dp.

4.2 Calibration curve between counts per second (cts) and kU_A/l using sandwichTRF

The calibrated line of two unit method were also studied in the same allergic patient and the healthy subject. As shown in Figure 3, the solid line represented the values of counts compared with kU_A/l. In allergic pateint, the highest specific IgE levels detected with Df, rDer f 2 and Dp were with 3.49 kU_A/l compared with 13456 cts, 3.49 kU_A/l compared with 12549 cts and 4.55 kU_A/l compared 33064 cts, respectively. The lowest specific IgE levels detected with Df, rDer f 2 and Dp were 0.22 kU_A/l compared with 1327 cts, 0.22 kU_A/l compared with 1311 cts and 0.28 kU_A/l compared with 2789 cts, respectively. In a healthy subject, the highest specific IgE levels after detection with all HDM allergens was less than 0.35 kU_A/l compared with 995 cts. The equation of specific IgE levels in both subjects between cts and kU_A/l unit was 10(906+3659cts) for Df, 10(855+3399cts) for rDer f 2 and 10(1122+7178cts) for Dp.

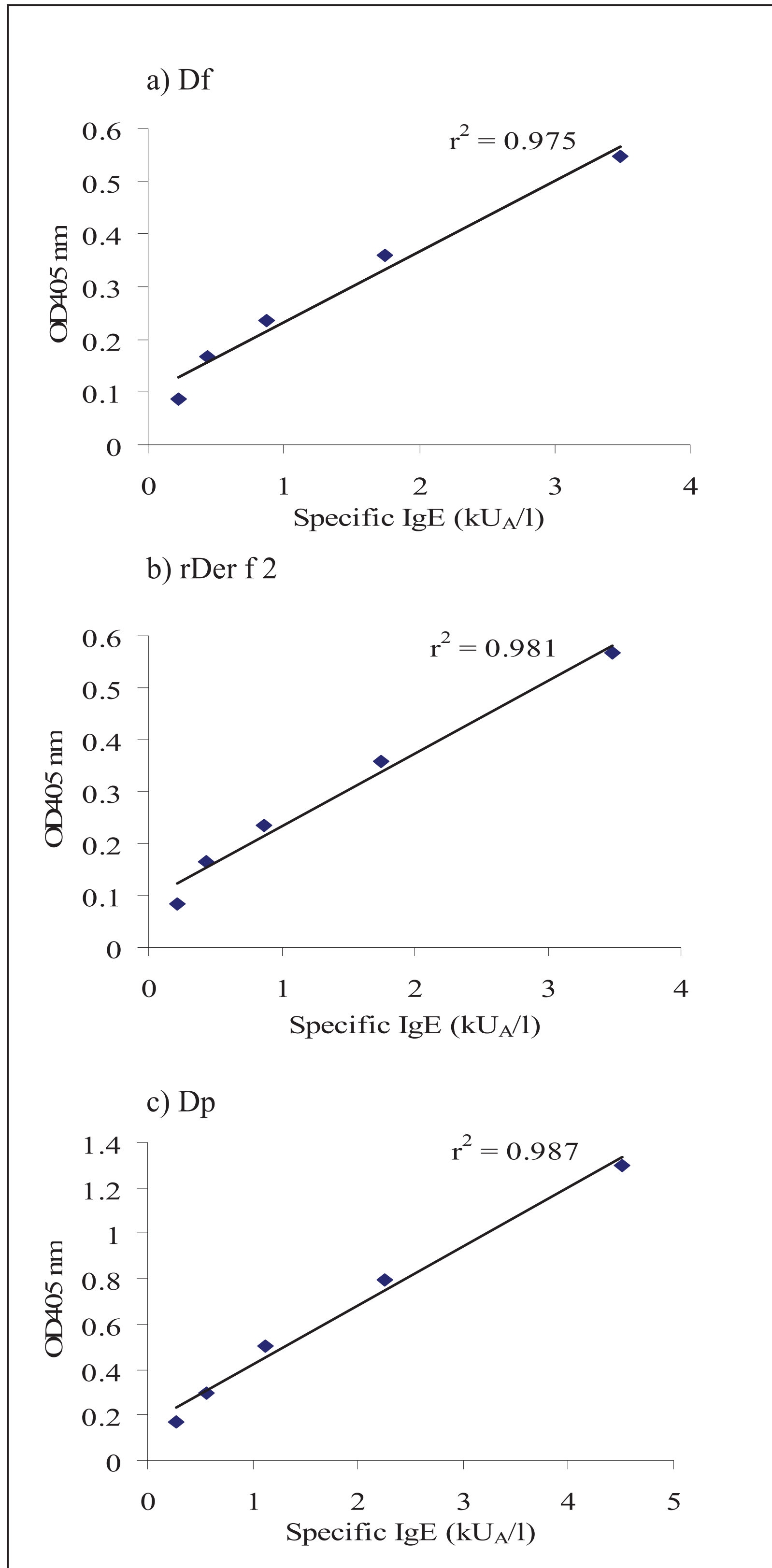

Figure 2 Calibration curve for sandwichELISA for a) Df, b) rDer f 2 and c) Dp.

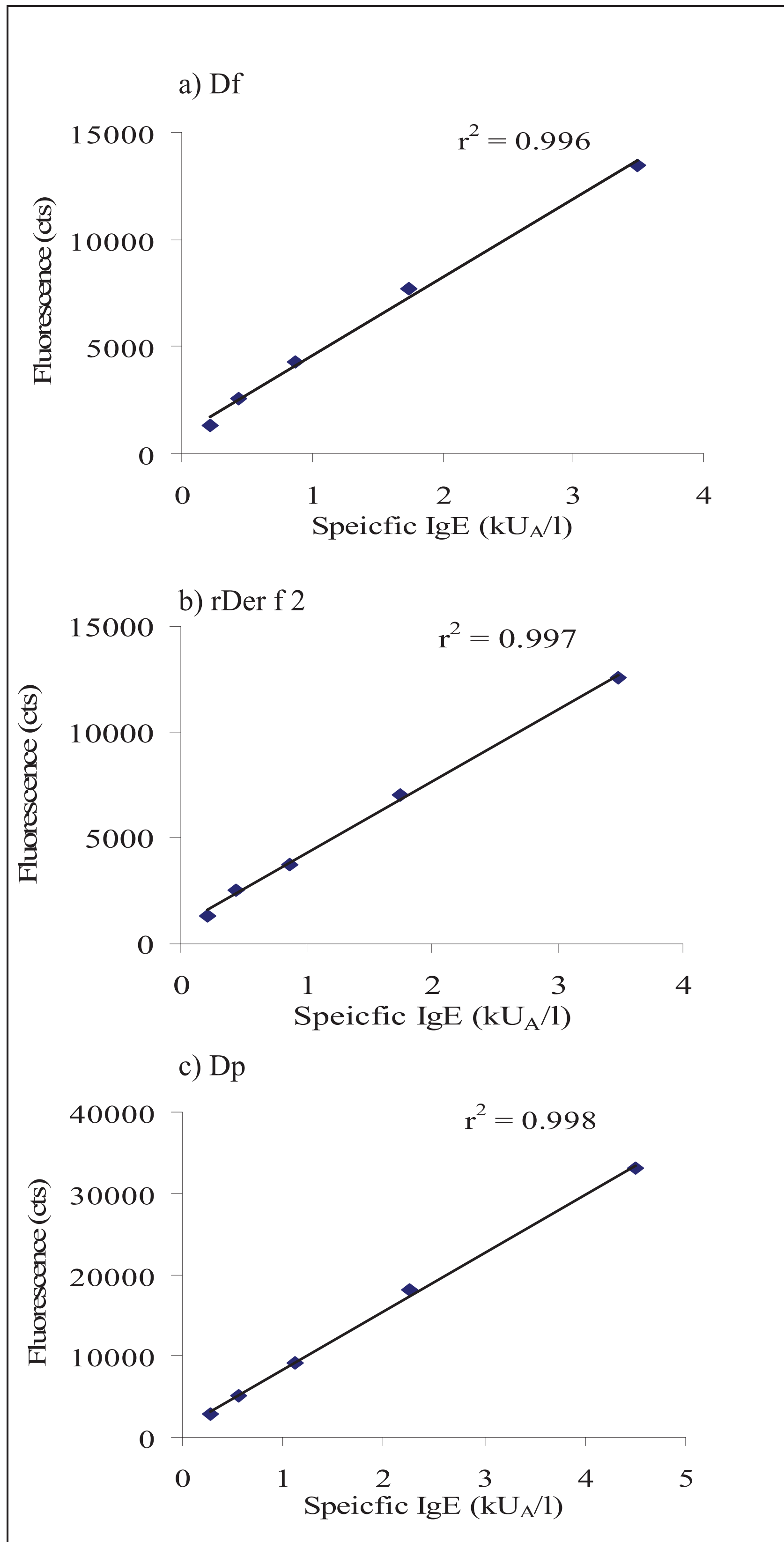

Figure 3 Calibration curve for sandwichTRF for a) Df, b) rDer f 2 and c) Dp.

5. Measurement of specific IgE to HDM allergens

5.1 Specific IgE to HDM allergens using sandwichELISA

Fifty subjects who showed allergic patients to commercial HDM and 19 healthy subjects were studied. Df, rDer f 2 and Dp allergens compared with pPICZ were used for specific IgE detection in both groups. As shown in Table 8 and 9, number of positive samples from sandwichELISA in allergic patients and healthy subject sera using Df, rDer f 2 and Dp allergen against group II mAb. Of 50 allergic patients, 13, 16 and 11 subjects showed specific IgE reactivity to Df (0.71-52.09 kU_A/l), rDer f 2 (0.86-54.27 kU_A/l) and Dp (3.07-76.39 kU_A/l), respectively.

Of 19 healthy subjects, only one subject showed specific IgE to rDer f 2 (0.85 kU_A/l). No detectable specific IgE to pPICZ was observed in sandwichELISA and sandwichTRF from all subjects.

5.2 Specific IgE to HDM allergens using sandwichTRF

Fifty patients who showed allergic reaction to commercial HDM and 19 healthy subjects were studied. Df, rDer f 2 and Dp allergens compared with pPICZ were used for specific IgE detection in both groups. As shown in Table 8 and 9, number of positive samples from sandwichTRF in allergic patient and healthy participant serum using Df, rDer f 2 and Dp allergen against group II mAb. Of 50 allergic patients, 31, 31 and 25 subjects showed specific IgE reactivity to Df (1.15-90.61 kU_A/l), rDer f 2 (1.27-79.82 kU_A/l) and Dp (0.67-106.17 kU_A/l), respectively.

Of 19 healthy subjects, 5, 6 and 1 subjects showed specific IgE reactivity to Df (1.52-2.14 kU_A/l), rDer f 2 (1.14-2.19 kU_A/l) and Dp (1.04 kU_A/l). No detectable specific IgE to pPICZ was observed in sandwichELISA and sandwichTRF from all subjects.

Number of positive samples from both methods in allergic patients and healthy subject sera using Df, rDer f 2 and Dp allergens against group II mAb were shown in Table 10. SandwichTRF detected specific IgE in higher percentage of allergic patient serum sample when tested using Df, rDer f 2 and Dp allergen (62, 62 and 50%) compared to sandwichELISA result (26, 32 and 22%). In 19 healthy subjects, sandwichELISA detected specific IgE in 0, 5.3 and 0% samples tested by the three HDM allergens while sandwichTRF found 26.3, 31.6 and 5.3% positive samples respectively.

SandwichTRF was compared to sandwichELISA by parallel testing of 50 serum specimens from rhinitis patients utilizing Df, rDer f 2 and Dp allergen respectively. Among allergic patients very good overall correlation between sandwich ELISA and sandwichTRF was found (Fig. 4). The linear regression coefficient values of Df, rDer f 2 and Dp calculated from the positive specimens varied between 0.93-0.96.

SandwichTRF positive cases from allergic patient sera tested with all HDM allergens were separated into two groups, namely those from sandwichELISA positive and sandwichELISA negative samples. Number of samples, frequency and median of each group was shown according to specific allergen in Table 11. Median, standard deviation, 25 % and 75% values were presented in Figure 5. Among sandwichTRF positive samples, 54.84 %, 48.39 % and 56 % of Df, rDer f 2, and Dp were identified as sandwichELISA negative samples.

Concentrations of HDM IgE TRF from all HDM allergens testing (allergic patients and healthy subjects) 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 HDM allergens did not give a statistically significant result in TRF technique.

Table 8 Profiles of 50 allergic patients detected specific IgE to Df, rDer f 2, Dp and pPICZ (cut-off value=0.35 kU_A/l).

ID.	Specific IgE (kU_A/l)							
	sandwichELISA				sandwichTRF			
	Df	rDer f 2	Dp	pPICZ	Df	rDer f 2	Dp	pPICZ
A1	0.71	1.20	-	-	2.77	2.15	1.16	-
A2	-	-	-	-	1.15	1.48	-	-
A3	-	-	-	-	-	5.01	-	-
A4	-	0.96	-	-	1.57	2.12	-	-
A5	0.71	-	-	-	2.72	1.93	0.67	-
A6	-	-	-	-	5.56	4.17	1.64	-
A7	-	1.06	-	-	-	2.38	0.88	-
A8	-	-	-	-	1.27	2.96	1.51	-
A9	5.09	12.93	7.73	-	6.22	13.22	12.81	-
A10	-	-	-	-	1.30	3.84	1.02	-
A11	10.32	14.46	9.76	-	16.10	44.46	22.90	-
A12	-	-	-	-	1.38	1.90	-	-
A13	1.98	4.27	3.07	-	5.77	7.01	6.60	-
A14	-	-	-	-	-	-	2.50	-
A15	23.71	41.65	34.66	-	77.59	98.26	54.95	-
A16	-	-	-	-	-	-	-	-
A17	-	-	-	-	2.75	2.45	1.30	-
A18	-	-	-	-	1.81	-	-	-
A19	-	-	-	-	-	-	-	-
A20	-	-	-	-	11.00	5.80	3.68	-
A21	-	-	-	-	3.18	3.63	1.93	-
A22	-	1.13	-	-	2.57	3.96	1.67	-
A23	5.16	21.98	25.28		2.88	14.18	17.94	-

- = < 0.35 kU_A/l

Table 8 (continue) Profiles of 50 allergic patients detected specific IgE to Df, rDer f 2, Dp and pPICZ (cut-off value=0.35 kU_A/l).

ID.	Specific IgE (kU_A/l)							
	sandwich ELISA				sandwich TRF			
	Df	rDer f 2	Dp	pPICZ	Df	rDer f 2	Dp	pPICZ
A24	-	-	-	-	3.52	5.42	3.07	-
A25	-	-	-	-	1.18	1.27	-	-
A26	27.64	46.75	49.76	-	10.63	30.45	46.16	-
A27	-	-	-	-	-	-	-	-
A28	34.82	45.04	69.64	-	17.54	19.15	74.92	-
A29	-	-	-	-	-	-	-	-
A30	12.49	21.63	22.39	-	26.59	27.87	28.73	-
A31	-	-	-	-	-	-	-	-
A32	6.88	6.91	29.95	-	29.85	29.82	48.95	-
A33	-	-	-	-	1.72	2.34	-	-
A34	-	-	-	-	1.16	1.77	-	-
A35	-	-	-	-	-	-	-	-
A36	-	-	-	-	-	-	-	-
A37	-	-	-	-	-	-	-	-
A38	-	-	-	-	-	-	-	-
A39	-	-	-	-	-	-	-	-
A40	-	-	-	-	-	-	-	-
A41	-	-	-	-	-	-	-	-
A42	28.05	30.53	39.58	-	38.05	32.85	37.19	-
A43	52.09	54.27	76.39	-	90.61	79.82	106.17	-
A44	-	-	-	-	-	-	-	-
A45	-	-	-	-	-	-	-	
A46	-	-	-	-	-	-	-	-

- = $<$ 0.35 kU_A/l

Table 8 (continue) Profiles of 50 allergic patients detected specific IgE to Df, rDer f 2, Dp and pPICZ (cut-off value=0.35 kU_A/l).

ID.	Specific IgE (kU_A/l)							
	sandwichELISA				sandwichTRF			
	Df	rDer f 2	Dp	pPICZ	Df	rDer f 2	Dp	pPICZ
A47	-	-	-	-	-	-	-	-
A48	-	-	-	-	2.42	-	-	-
A49	-	-	-	-	2.33	2.74	6.08	-
A50	-	0.86	2.17	-	5.68	6.41	12.52	-

- = < 0.35 kU_A/l

Table 9 Profiles of 19 Healthy subjects detected specific IgE to Df, rDer f 2, Dp and pPICZ (cut-off value=0.35 kU_A/l).

ID.	Specific IgE (kU_A/l)							
	sandwichELISA				sandwichTRF			
	Df	rDer f 2	Dp	pPICZ	Df	rDer f 2	Dp	pPICZ
H1	-	0.85	-	-	1.52	1.55	-	-
H2	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	1.50	-	-
H4	-	-	-	-	1.36	1.92	-	-
H5	-	-	-	-	1.68	-	-	-
H6	-	-	-	-	2.14	-	-	-
H7	-	-	-	-	-	1.14	-	-
H8	-	-	-	-	1.69	2.19	-	-
H9	-	-	-	-	-	-	-	-
H10	-	-	-	-	-	1.25	1.04	-
H11	-	-	-	-	-	-	-	-
H12	-	-	-	-	-	-	-	-
H13	-	-	-	-	-	-	-	-
H14	-	-	-	-	-	-	-	-
H15	-	-	-	-	-	-	-	-
H16	-	-	-	-	-	-	-	-
H17	-	-	-	-	-	-	-	-
H18	-	-	-	-	-	-	-	-
H19	-	-	-	-	-	-	-	-

- = < 0.35 kU_A/l

Table 10 Number of positive samples from sandwichELISA and sandwichTRF in allergic patient and healthy participant serum as seen using Df, rDer f 2 and Dp allergens against group II mAb.

Allergen tested	Number of positive samples: n (frequency %)	
	Allergic patients Total n = 50	Healthy participants Total n = 19
Df		
sandwichELISA	13 (26%)	0 (0%)
sandwichTRF	31[1] (62%)	5 (26.3%)
rDer f 2		
sandwichELISA	16 (32%)	1 (5.3%)
sandwichTRF	31[2] (62%)	6 (31.6%)
Dp		
sandwichELISA	11 (22%)	0 (0%)
sandwichTRF	25[3] (50%)	1 (5.3%)

[1, 2,] and [3] correspond to the same data in Table 11.

Table 11 SandwichTRF positive cases in serum from allergic patients categorized by sandwichELISA result in Df, rDer f 2 and Dp testing.

HDM allergens	sandwichTRF positive cases in symptomatic patients	
	sandwichELISA positive	sandwichELISA negative
Df: total 31 samples[1]		
n (frequency %)	13 (45.16%)	17 (54.84%)
Median (kU_A/l)	13.37	1.81*
rDer f 2: 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; $p < 0.001$*

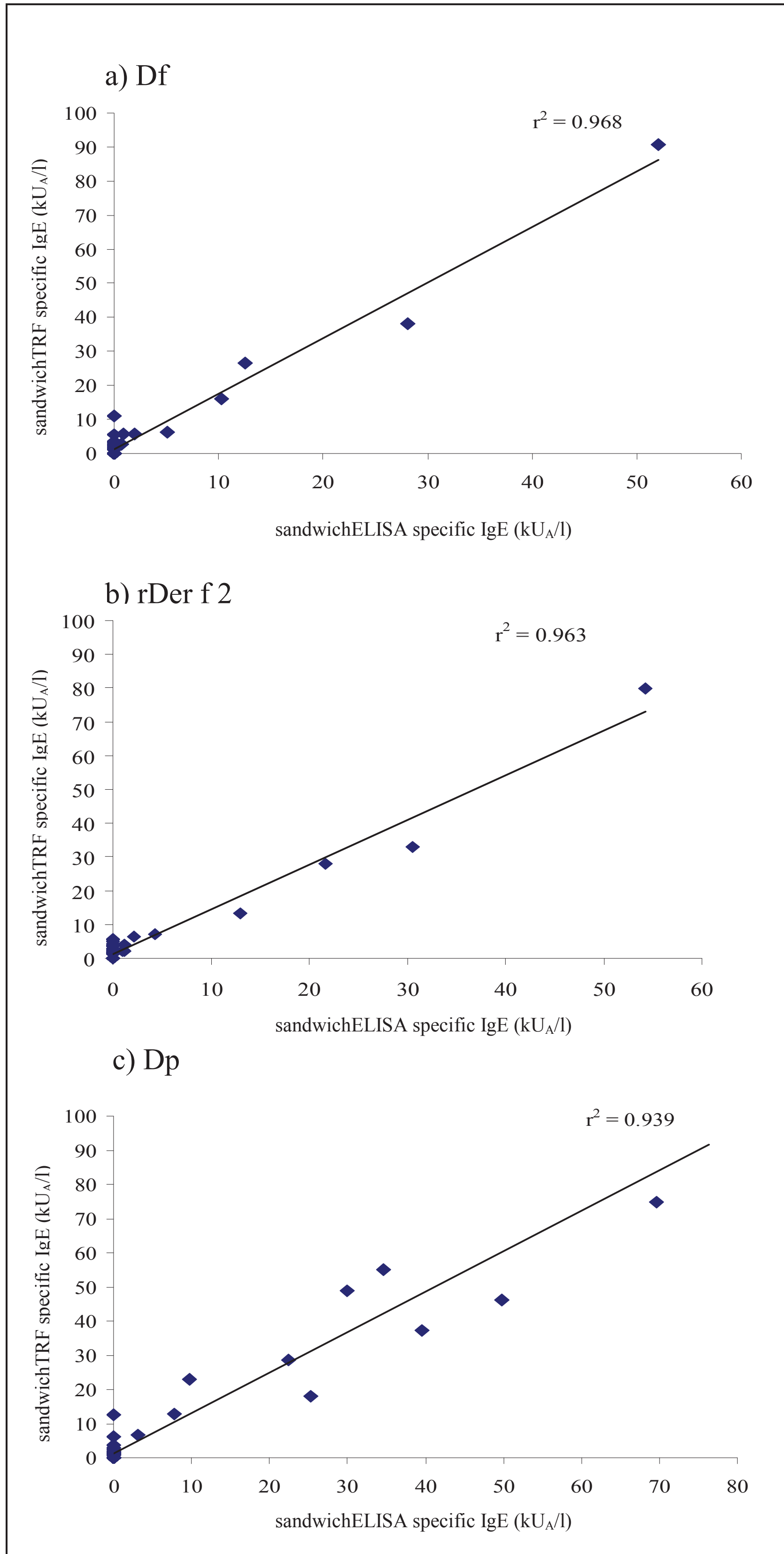

Figure 4 Correlation between levels of specific IgE against a) Df, b) rDer f 2 and c) Dp measured by sandwichELISA and sandwichTRF.

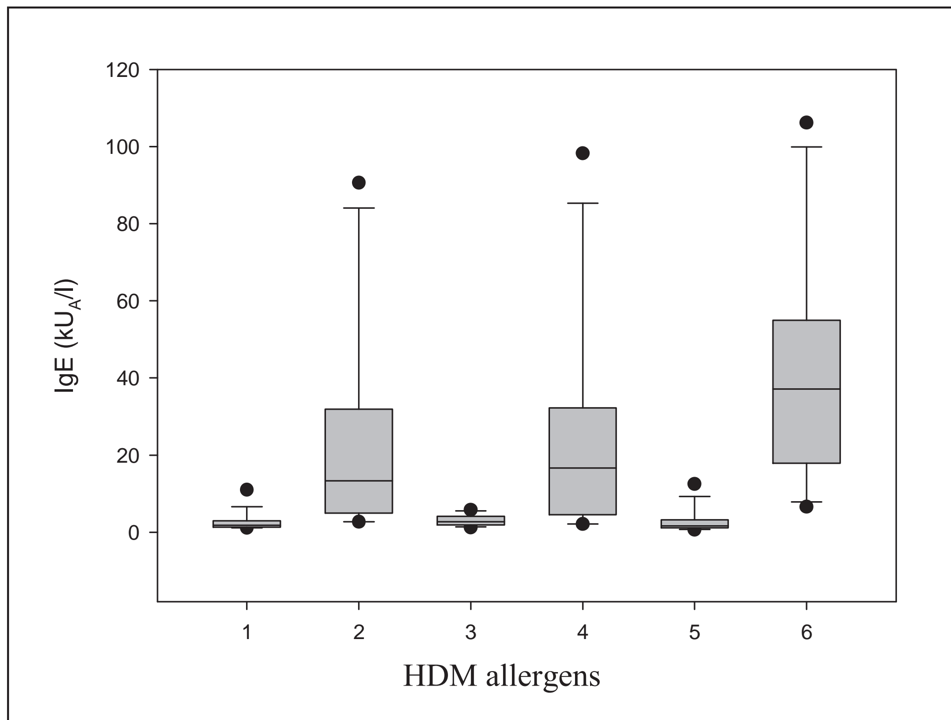

Figure 5 SandwichTRF positive cases from serum of allergic patients tested with all three HDM allergens. Median, standard deviation, 25 and 75 percent values were presented. HDM allergens, Df : 1) sandwichELISA negative, 2) sandwichELISA positive; rDer f 2 : 3) sandwichELISA negative, 4) sandwichELISA positive; Dp : 5) sandwichELISA negative, 6) sandwichELISA positive.

CHAPTER V

DISCUSSION

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 group II mAb showed one band of 14 kDa (Figure 1), agreeing with previous reports Thus it is an implication that rDer f 2 corresponded to *D. farinae* (58, 78). It is known that *D. farinae* usually coexists with *D. pteronyssinus*. As a result the assay measuring IgE binding to rDer f 2 would not distinguish *D. farinae* sensitizationwith cross reactivity (127).

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 subjects (n = 19) was determined by three different allergens, namely Df, rDer f 2 and Dp measured by TRF compared with ELISA.

As shown in Table 10, the efficacy of recombinant antigen (rDer f 2) and natural antigen (Df or Dp) were examined in allergic patients and non-allergic individuals by both TRF and ELISA. In allergic patients, we found that only 26 %, 32 % and 22 % result was obtained by ELISA, while 62 %, 62 % and 50 % of patient sera reacted with Df, rDerf2, and Dp respectively using TRF. The increase in rate of HDM IgE detection was significant at 34 %, 30 % and 28 % for all three allergen, respectively. This is the first indication suggesting that TRF 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 % positive samples in healthy sera, TRF detected 26.3 %, 31.6 % and 5.3 % positive samples. That is the 26.3 %, 21.3 % and 5.3 % 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 TRF, 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 TRF and

ELISA. TRF showed 10 %, 2 % and 0 % cross-reactivity between Df/rDer f 2, rDer f 2/Dp and Df/Dp, while ELISA result showed 2 %, 0 % and 0 %, 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/rDer f 2 and rDer f 2/Dp. These numbers confirms TRF sensitivity over ELISA and suggests that rDerf2 is a slightly better antigen as seen from cross-reactivity data.

Table 11 and Figure 5 revealed lower detection limit and the sensitivity of TRF. All HDM allergens provided significantly different median between ELISA positive and negative samples in Mann-Whitney Rank Sum Test with $p < 0.001$. Figure 5 showed 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. TRF 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 rDer f 2. Result from the test for several related samples using Friedman test showed that TRF results using all three allergens were not significantly different from another. Thus this investigation suggested that rDer f 2 can be used as a HDM allergen in TRF. Since rDer f 2 has been expressed in large quantity, this should ensure a homogeneous batch supply of HDM allergen for time to come. Immunoassays based on time-resolved fluorometry represented 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 (76, 131). 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 (33).

The time-resolved immunofluorometric assay developed in this investigation provided sensitive and highly specific techniques for the determination of HDMspecific IgE in human sera. Measurements achieved with TRF and ELISA were

highly correlated (r2 between 0.93-0.96). In regard to the limit of detection, the assay allowed the detection of HDM-specific IgE as low as < 0.35 kUA/l, which was well below limits of detection reported by other techniques, namely Immunoblot, ELISA, and RIA. TRF could therefore be considered the most sensitive method for quantifying HDM-specific IgE. In addition, the assay range of TRF was larger than that of conventional ELISA in our laboratory. This finding was consistent with previous reports (124, 90). It is clearly seen that TRF showed sensitivity to serum of allergic patients with symptom whose serum were formerly HDM IgE negative using ELISA. TRF showed IgE detection in real samples from ELISA HDM positive allergic patients. TRF was also capable of detecting IgE in ELISA HDM negative but positive to SPT aeroallergens. It is thus demonstrated that a broad range of HDM-specific IgE could be detected with the TRF.

CHAPTER VI

CONCLUSION

TRF was developed for HDM allergen IgE testing and evaluated. Such improvements may be useful for screening of HDM allergy and other aeroallergens. TRF of Df, rDer f 2 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, rDer f 2, was proven to be as good an allergen as its natural counterpart, namely Df and Dp. It is anticipated that TRF 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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APPENDIX

APPENDIX A

List of Abbreviations

Abbreviation	Term
A	Allergic
APCs	Antigen presenting cells
BMGY	buffered minimal glycerol complex medium
BMMY	buffered minimal methanol complex medium
Bt	*Blomia tropicalis*
BSA	bovine serum albumine
CR	American Cockroach
°C	degree Celsius
cm	centimeter
cts	counts per second
DAS-TR-IFMA	Double-antigen Sandwich Time-resolved immunofluorometric assay
Der	*Dermatophagoides*
Df	*Dermatophagoides farinae*
Dp	*Dermatophagoides pteronyssinus*;
ELISA	Enzyme Link Immuno Sorbent Assay
EU	europium
et al	et alia
F	Female
FPA	Fluorescence polarization assay
H	Healthy
HDM	House Dust Mite
h	hour (s)
IL	interleukin
IFN	interferon
IUIS	International Union of Immunological Societies
Ig	Immunoglobulin
kDa	kilo Dalton
kU_A/l	kilo units of aprotinin-specific antibodies per liter

Abbreviation	Term
M	Male
mAb	monoclonal antibody
mg	milligram
min	minute (s)
ml	milliliter
mm	millimeter
µg	microgram
µl	microliter
nm	nanometer
OD	optical density
P. pastoris	*Pichia pastoris*
PBS	phosphate buffer saline
rpm	round per minute
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel Electrophoresis
SPT	Skin prick Test
SMM	Spent Mite Medium
TRF	Time-resolved Fluoroimmunoassay
TR-FRET	Time-resolved Fluorescent resonance energy transfer
TR-IFMA	Time-resolved Immunofluorometric assay
V	volt
VEGF	vascular endothelial growth factor
v/v	volume / volume
w/v	weight / volume
YPD	yeast extract peptone dextrose

APPENDIX B

General Reagents

Phosphate Buffered Saline (PBS), pH 7.4

NaCl	8.0	g
KH_2PO_4	0.24	g
Na_2HPO_4	1.44	g
KCl	0.20	g

Dissolve in deionized water, adjust to pH 7.4 with HCl and adjust to final volume of 1,000 ml with deionized water. Sterized by autoclave.

Culture Media

Yeast Extract Peptone (YP)

yeast extract	1.0	g
tryptone	2.0	g

Dissolve and adjust the volume of 90 ml with deionized water. Sterized by autoclave at 121 °C, 15 lb/square inches for 15 min.

10X Dextrose (D)

Dextrose	5.0	g

Dissolve and adjust the volume of 25 ml with deionized water. Sterized by autoclave.

Buffered minimal (BM)

yeast extract	5.0	g
tryptone	10.0	g

Dissolve and adjust the volume of 350 ml with deionized water. Sterized by autoclave.

1 M K_2HPO_4

K_2HPO_4	8.709	g

Dissolve and adjust the volume of 50 ml with deionized water.

1M KH_2PO_4

KH_2PO_4	13.609	g

Dissolve and adjust the volume of 100 ml with deionized water.

1M Potassium Phosphate Buffer, pH 6.0

1 M K_2HPO_4	13.2	ml

1M KH_2PO_4	86.8	ml

Dissolve in deionized water, adjust to pH 6.0 and sterized by autoclave at 121 °C, 15 lb/square inches for 15 minutes.

10X 13.4% Yeast Nitrogen Base with $(NH_4)_2SO_4$ but without amino acid (YNB)

YNB	13.4	ml

Dissolve and adjust the volume of 100 ml with deionized water. Sterized by 0.22 μ filter and store at night.

10X Glycerol

Glycerol	5.0	ml

Dissolve and adjust the volume of 45 ml with deionized water. Sterized by autoclave.

10X Methanol

Methanol	3.0	ml

Dissolve and adjust the volume of 97 ml with deionized water. Sterized by autoclave.

500X Biotin

Biotin	2.0	g

Dissolve and adjust the volume of 10 ml with deionized water. Sterized by 0.22 μ filter and store at night.

Yeast extract peptone dextrose (YPD)

YP	9.0	ml
10X D	1.0	ml
Zeocin™ (100 μg/ml)	10.0	μl

Buffered minimal glycerol complex medium (BMGY)

BM	70.0	ml
1M Potassium Phosphate Buffer	10.0	ml
10X YNB	10.0	ml
10X GY	10.0	ml
500X B	100.0	μl

Buffered minimal methanol complex medium (BMMY)

BM	70.0	ml

1M Potassium Phosphate Buffer	10.0	ml
10X YNB	10.0	ml
10X M	10.0	ml
500X B	100.0	µl

Reagent for Polyacrylamine Gel Electrophoresis

Lower gel buffer

Tris base	18.2	g
Sodium dodecyl sulfate (SDS)	0.4	g

Dissolve in deionized water, adjust to pH 8.8 and adjust to final volume of 100 ml with deionized water.

Upper gel buffer

Tris base	6.0	g
SDS	0.4	g

Dissolve in deionized water, adjust to pH 6.8 and adjust to final volume of 100 ml with deionized water.

10% Ammonium persulfate (APS) (w/v)

Ammonium persulfate	1.0	g
Deionized water	10.0	ml

12.5% Resolving gel

Lower gel buffer	1.0	ml
Deionized water	1.33	ml
30% Acrylamide	1.67	ml
10% APS	20.0	µl
TEMED	8.0	µl

4% Resolving gel

Upper gel buffer	0.78	ml
Deionized water	1.83	ml
30% Acrylamind	0.39	ml
10% APS	20.0	µl
TEMED	5.0	µl

4X Sample buffer

Glycerol	4.0	ml
SDS	0.4	g
Upper gel buffer	5.0	ml
Bromophenol blue	2.0	g
2- Mercaptoethanol	1.0	ml

Dissolve and adjust the volume of 10 ml with deionized water.

10X Electrode buffer

Tris base	30.0	g
Glycine	144.0	g
SDS	10.0	g

Dissolve in deionized water, adjust to pH 8.3 and adjust to final volume of 1,000 ml with deionized water.

Working Electrode buffer

10X Electrode buffer	100	ml

Dissolve and adjust the volume of 900 ml with deionized water.

Coomassie Brilliant Blue (CBB)

CBB-G 250 stock	5	g]
Ammonium sulfate	50	g
85% O-phosphoric acid (w/w)	6	ml
Methanol	125	ml

Dissolve and adjust to final volume of 500 ml with deionized water.

5% SDS

SDS	0.25	g

Dissolve and adjust to final volume of 5 ml with deionized water.

7.5X Electroblotting buffer

Tris base	4.5375	g
Glycine	21.6	g

Dissolve and adjust to final volume of 200 ml with deionized water.

Working Electroblotting buffer

7.5X Electroblotting buffer	200	ml
Methanol	300	ml
5% SDS	3	ml

Dissolve and adjust to final volume of 1500 ml with deionized water.

0.1 % PBS-Tween 20 (PBS-T)

PBS	1,000	ml
Tween 20	1	ml

Blocking/Diluting buffer (PBST with 3% BSA)

PBS-T	100	ml
BSA	3	g

Reagent for Enzyme-Linked Immunosorbent asaay

50mM carbonate/bicarbonate buffer, pH 9.6

Na_2CO_3	1.59	g
$NaHCO_3$	2.93	g

Dissolve in deionized water, adjust pH to 9.6 and adjust to 1,000 ml final volume with deionized water. Sterized by autoclave.

0.05% PBS-Tween (PBS-T)

PBS	1,000	ml
Tween 20	0.5	ml

Blocking/Diluting buffer (PBST with 1% BSA)

PBS-T	100	ml
BSA	1	g

APPENDIX C

ข้อมูลสำหรับผู้เข้าร่วมโครงการวิจัย

(Patient/Participant Information Sheet)

ชื่อโครงการ การตรวจวัดระดับ IgE ต่อสารก่อภูมิแพ้จากไรฝุ่นด้วย time-resolved fluoroimmunoassay

การตรวจวินิจฉัยหาสาเหตุของการเกิดโรคภูมิแพ้ของผู้ป่วยสามารถทำได้หลายวิธี เช่น การทดสอบภูมิแพ้ทางผิวหนังของผู้ป่วยโดยตรงด้วยวิธี skin test พบว่ามีความไวสูงแต่ไม่จำเพาะเจาะจงขณะที่การตรวจวัดระดับ IgE ด้วยวิธี ELISA test มีความไวต่ำกว่าวิธีแรกแต่มีความจำเพาะเจาะจงมากกว่า จากรายงานของต่างประเทศพบว่า time-resolved fluoroimmunoassay มีความไวและความจำเพาะเจาะจงสูง ด้วยเหตุนี้ผู้วิจัยจึงต้องการที่จะพัฒนาเทคนิคนี้ขึ้นในประเทศไทย เพื่อเป็นประโยชน์ต่อผู้ป่วยในการวินิจฉัยโรคภูมิแพ้ต่อไป

วิธีดำเนินการแพทย์ผู้ดูแลรักษาจะทำการซักประวัติ ตรวจร่างกาย วัดความดันโลหิตชีพจร และเก็บตัวอย่างเลือดจากผู้ป่วยโดยการเจาะเส้นเลือดดำบริเวณข้อพับแขนปริมาณ 10 มิลลิลิตร ตามวิธีมาตรฐานการดำเนินการเจาะเลือด

เด็กในปกครองของท่านหรือตัวท่านเองจะได้รับการดูแลในโรงพยาบาลเป็นเวลาประมาณ 30 นาที หลังได้รับการเจาะเลือด โดยไม่เสียค่าใช้จ่าย และพิจารณาให้กลับบ้านได้หากไม่พบอาการผิดปกติ

ในระหว่างการดำเนินการเจาะเลือด เด็กหรือตัวท่านเองจะได้รับการดูแลอย่างใกล้ชิด กรณีที่ท่านมีข้อสงสัยขอให้ท่านติดต่อสอบถามแพทย์ผู้ดำเนินการเจาะเลือดได้ทันที

การยินยอมเข้าร่วมโครงการนี้เป็นไปโดยความสมัครใจ ท่านสามารถปฏิเสธ หรือขอยกเลิกการเข้าร่วมโครงการได้ทุกเวลา

ข้อมูลที่ได้จากโครงการนี้ จะถูกจัดเก็บไว้ในคอมพิวเตอร์อย่างน้อย 15 ปี และจะไม่มีการระบุชื่อผู้ที่เข้าร่วมโครงการ มีเพียงแพทย์เท่านั้นที่จะรู้ว่าข้อมูลใดเกี่ยวข้องกับเด็กในกรณีที่มีการตีพิมพ์ผลของการเจาะเลือดในวารสารทางการแพทย์และวารสารอื่น ๆ ในระดับนานาชาติ เพื่อเป็นการรักษาความลับจะไม่มีการเปิดเผยชื่อเด็กในปกครองของท่าน

ในกรณีที่ท่านมีข้อสงสัยเกี่ยวกับโครงการ กรุณาติดต่อ แพทย์หญิง นาเรศ วงศ์ไพฑูรย์ หรือ แพทย์หญิง วรวรรณ ดุรงค์พิศิษฏ์กุล ได้ที่ งานกุมารเวชกรรม โรงพยาบาลตำรวจ หมายเลขโทรศัพท์ 0-2517-4270-9 ต่อ 1459 หมายเลขโทรสาร 0-2652-5033 และ/หรือ อาจารย์ ดร. จันทร์ดี ระแบบเลิศ หมายเลขโทรศัพท์ 084-7102575 ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศิลปากร วิทยาเขตพระราชวังสนามจันทร์ จ.นครปฐม

ใบยินยอมเข้าร่วมโครงการวิจัย
(Informed Consent Form)

ชื่อโครงการ การตรวจวัดระดับ IgE ต่อสารก่อภูมิแพ้จากไรฝุ่นด้วย time-resolved fluoroimmunoassay

ข้าพเจ้า...(บิดา มารดา/ผู้ปกครอง) ที่อยู่... หมายเลขโทรศัพท์...ได้อ่านเอกสารรายละเอียดโครงการที่มีชื่อข้างต้น และได้มีโอกาสซักถามเกี่ยวกับโครงการนี้จาก แพทย์หญิงนาเรศ วงศ์ไพฑูรย์ หรือ แพทย์หญิงวรวรรณ ดุรงค์พิศิษฏ์กุล และเข้าใจโครงการนี้

ข้าพเจ้ายินยอมให้...(บุตร/ธิดา/ตนเอง) อายุ..................ปี เลขที่โรงพยาบาล..เข้าร่วมในโครงการ

หากข้าพเจ้ามีข้อสงสัยเกี่ยวกับการดำเนินการเจาะเลือด ข้าพเจ้ามีสิทธิซักถามแพทย์ได้ในระหว่างการดำเนินการเจาะเลือดและคำชี้แจงของแพทย์ผู้ดำเนินการเจาะเลือดยังไม่เป็นที่เข้าใจ ข้าพเจ้ามีสิทธิแจ้งต่อประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์ หมายเลขโทรศัพท์ 0-2252-8111-25 ต่อ 4135 หรือผู้บังคับการโรงพยาบาลตำรวจ หมายเลขโทรศัพท์ 0-2252-8111-25 ต่อ 4222 ได้ และหากข้าพเจ้าไม่พอใจในการดำเนินการเจาะเลือด ข้าพเจ้ามีสิทธิปฏิเสธการดำเนินการเจาะเลือดวิธีนี้ได้ทันที โดยไม่เสียสิทธิในการรักษาในโรงพยาบาลต่อไป

ข้าพเจ้าได้อ่านและเข้าใจเกี่ยวกับการดำเนินการเจาะเลือดทั้งหมดตามคำอธิบายข้างต้นแล้ว ข้าพเจ้ายินยอมรับการดำเนินการเจาะเลือดตามวิธีดังกล่าว

....

ลายเซ็นของบิดา มารดา/ผู้ปกครอง/ตนเอง วัน/เดือน/ปี

....

ลายเซ็นของพยาน วัน/เดือน/ปี

....

ลายเซ็นของพยาน วัน/เดือน/ปี

....

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Working Experience

2005-2007	Staff in the project house dust mite, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University
2010-present	Business Development staff at Bank for Agriculture and Agricultural Co-operatives