

Quantification of Fel d 1 in house dust samples of cat allergic patients by using monoclonal antibody specific to a novel IgE-binding epitope

Natt Tasaniyananda,^{1,2} Anchalee Tungtrongchitr,^{2,3} Watee Seesuy,² Yuwaporn Sakolvaree,² Pisinee Aiumurai,^{2,4} Nitaya Indrawattana,⁵ Wanpen Chaicumpa,^{2,3} Nitat Sookrung^{2,4}

Abstract

Background: Avoidance of allergen exposure is an effective measure for preventing naïve and allergic individuals from sensitization (primary intervention) and disease aggravation (secondary intervention), respectively. Regular monitoring of the allergens in the environment is required for the effective intervention. Thus, there is a need for cost-effective test kits for environmental allergen quantifications.

Objective: To invent a test kit for quantification of cat major allergen, Fel d 1.

Methods: A mouse monoclonal antibody (MAb) specific to the newly identified IgE-binding conformational epitope of the cat major allergen (Fel d 1) and rabbit polyclonal IgG to recombinant Fel d 1 were used as allergen capture and detection reagents, respectively. Native Fel d 1 was used in constructing a standard curve.

Results and Conclusion: Sixteen of 36 dust samples collected from houses of cat allergic subjects in Bangkok contained Fel d 1 above 0.29 µg/gram of dust which is considered as a novel threshold level for causing cat allergy sensitization or symptoms. Among them, 7 samples contained the allergen exceeding 2.35 µg/gram of dust which is the level that would aggravate asthma. Results of the allergen quantification using the locally made test kit showed strong correlation ($r = 0.923$) with the allergen quantification using commercialized reagents. The assay using MAb to Fel d 1 IgE-binding epitope of this study has potential application as an economic and practical tool for cat allergy intervention measure especially in localities where health resources are relatively limited.

Keywords: Allergen quantification, allergy, cat allergen, Fel d 1, IgE-binding epitope, sandwich ELISA

From:

¹ Graduate Program in Immunology, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

² Center of Research Excellence on Therapeutic Proteins and Antibody Engineering, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

³ Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

⁴ Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

⁵ Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand

Corresponding author:

Nitat Sookrung
Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University,
2 Wanglung Road, Bangkok-noi, Bangkok 10700, Thailand
E-mail: nitat.soo@mahidol.ac.th

Introduction

Several cat-derived proteins are allergens that are not only found indoor (houses of cat keepers), but also in the open space due to their airborne nature. The proteins can sensitize humans to a variety of allergic manifestations of which allergic rhinitis and atopic asthma are the most common clinical features.^{1,2} Presently, eight cat-derived proteins are officially recognized as human allergens by the IUIS Allergen Nomenclature Sub-committee.³⁻¹⁷ Among them, Fel d 1 (a uteroglobin protein) is regarded as a cat's major allergen as it sensitized more than 50% of the cat allergic subjects.^{12,17,18} Molecularly, this allergen is a 18-20 kDa protein consisting of disulfide-linked peptide chains 1 and 2.⁶ Individual peptide acquires four anti-parallel helical structures designated H1-H4 for the chain 1 and H5-H8 for the chain 2.⁶ Native Fel d 1 may form tetramers of about 35-40 kDa by non-covalent association of two heteromeric dimers.^{6,19} Previously, three

IgE-binding linear epitopes of the Fel d 1 molecule have been recognized.²⁰ These epitopes encompass residues 25-38 and 46-59 of the chain 1 and 15-28 of the chain 2, respectively.²⁰ Recently, we have generated a mouse hybridoma (clone C48) secreting monoclonal antibody (MAbC48) that impeded the binding of the cat allergic patient's serum IgE to the Fel d 1. Computerized simulation predicted that the MAb formed interface contact with a novel IgE-binding conformational epitope of the Fel d 1 formed by spatially juxtaposed residues in the chain 1, i.e., L34, T37, T39 P40, E42, E45, R61, K64, N65, D68, E73 and K76.²¹ While the novel IgE-binding epitope of Fel d 1 has potential applications for personalized component resolved diagnosis (CRD) and component resolved immunotherapy (CRIT),²²⁻²⁴ the specific MAb to the epitope is a useful reagent for specific detection and quantification of the Fel d 1 in environmental samples for cat allergy intervention measure. Thus in this study, the MAbC48 was used as Fel d 1 capture reagent for quantification of the cat major allergen in dust samples in comparison with the commercially available reagents. Efficacy of the locally invented test kit is reported herein.

Methods

Preparations of crude extracts of cat and dog hairs, American cockroach (Periplaneta americana) and house dust mites (Dermatophagoides pteronyssinus and D. farinae)

Hairs of healthy female cats (*Felis domesticus*) and dogs (*Canis familiaris*) that the owners brought to a veterinary clinic for spaying were obtained. To each gram of the hairs, 20 ml of phosphate buffered saline, pH 7.4 (PBS) containing 0.1% Tween-20 (PBST) were added. The preparations were sonicated (40 kHz) in ice-bath for 30 min before filtering through a cell strainer to remove coarse materials, then centrifuged (2,000 × g, 4°C, 30 min). The clear supernatants were dialyzed against distilled water at 4°C overnight. The hair extracts were concentrated and protein contents were determined.

Extracts of *P. americana* (American cockroach), house dust mites, i.e., *D. pteronyssinus* and *D. farinae* were prepared as described previously.²⁵⁻²⁷

Protein determination

A microtiter plate protocol of Bio-Rad Protein Assay (Bio-Rad, USA) based on Bradford's method was used for determining protein concentration of a preparation which was performed according to the instruction manual. Bovine serum albumin (BSA; Sigma-Aldrich, USA) was used for constructing a standard curve.

Preparation of recombinant Fel d 1

Recombinant Fel d 1 (rFel d 1) with 6× histidine tag was prepared from a transformed BL21 (DE3) *E. coli* carrying recombinant plasmid with a DNA insert coding for full-length Fel d 1 (chains 1 and 2 with Gly₄Ser₁ linker between the two chains) as described previously.²⁸ Appropriately transformed *E. coli* clone was grown in Luria-Bertani (LB) broth containing 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) as described previously.²¹ The bacterial pellet obtained after centrifugation was sonicated (30% amplitude/0.6 sec cycle)

in lysis buffer (4% glycerol in 10 mM Tris-HCl, pH 7.4) and centrifuged (15,000 × g, 4°C, 20 min). The recombinant protein was purified using Ni-NTA affinity resin (HisPur™ Ni-NTA resin, Thermo Scientific, USA) and the resin-bound protein was eluted from the affinity resin by using 250 mM imidazole in PBS. Protein concentration was determined.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE and protein staining with Coomassie Brilliant Blue G-250 (CBB) dye were performed to visualize the complexity of protein profile of a preparation. The electrophoresis was carried out using a 4% stacking gel and a 12% separating gel. The vertical slab gel (8.0 × 7.3 cm) was cast in Mini-PROTEAN®3 cell apparatus (Bio-Rad, California, USA). One part of protein sample to be resolved was mixed with five parts of 6× sample buffer [0.6 mg SDS, 0.5 mg bromophenol blue, 3.74 ml Tris-HCl (0.5 M, pH 8.8), 4.6 ml glycerol and 1.5 ml β-mercaptoethanol and distilled water to make 10 ml volume] and boiled for 5 min. After boiling, the preparation was centrifuged and the supernatant was loaded into a slot of the stacking gel. PageRuler™ pre-stained protein ladder (Fermentas, Lithuania) was included in at least one slot of each gel slab. Electrophoresis was done in electrophoresis chamber at 20 mA per gel with an electric power supply (PowerPac™, Bio-Rad). At the end of the electrophoretic run, the gel containing the separated proteins was stained with CBB dye. The gel was fixed with 2% O-phosphoric acid in 20% (v/v) methanol in distilled water for 1 h at 25°C. After discarding the fixing solution, the gel was placed in the CBB solution at 25°C overnight on a gentle rocking platform. Excess stain was removed and the gel was immersed in a destaining solution until the background was clear. The stained gel was either kept in 20% ammonium sulfate in distilled water at 4°C or photographed.

Preparation of rabbit polyclonal antibody (PAb) to rFel d 1

Rabbit PAb to rFel d 1 was prepared as described previously.²¹ The animal experiments received approval from the Siriraj Animal Ethic Committee, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (SI-ACUC 015/2557). Three doses of 500 µg of rFel d 1 mixed with alum adjuvant (Pierce, Thermo Scientific, MA, USA) were injected intramuscularly at two week-intervals into a New Zealand White rabbit. One week after the last dose, the animal was bled. Total serum immunoglobulin was precipitated out from the immune serum using 50% saturated ammonium sulfate and IgG (polyclonal antibody; PAb) was purified from the immunoglobulin preparation by using Protein A Sepharose Fast Flow resin (GE Healthcare, Buckinghamshire, UK). Protein content of the PAb was determined and standardized to the required concentration. Titer of PAb against the rFel d 1 was checked by means of indirect ELISA.²⁶

Monoclonal antibody specific to IgE-binding epitope of Fel d 1

Hybridoma clone C48 which secretes IgG1/kappa monoclonal antibody (MAbC48) to a novel conformational IgE-binding epitope of Fel d 1 was generated previously.²¹ The clone was grown in a serum-free medium to late log phase and the culture supernatant was harvested. Monoclonal IgG (MAb)

was purified from the cell spent medium by using HiTrap Protein-G HP column (GE Healthcare) and protein content was determined and adjusted to a desired concentration.

Preparation of native Fel d 1

Native Fel d 1 (nFel d 1) was used as a standard allergen in the Fel d 1 quantification test kit. The allergen was purified from the crude cat's hair extract (CE) by using affinity (MAbC48-bound) Sepharose resin.²¹ Briefly, one gram of CNBr-activated Sepharose™ 4B (GE Healthcare) was dispersed in 200 ml of 1 mM HCl for 15 min. After washing extensively with a coupling buffer (0.1 M NaHCO₃ containing 0.5 M NaCl), 30 mg of purified MAbC48 were mixed with the resin on a rotator at 25°C for 1 h. Unbound antibody was removed and the resin was washed alternately with the coupling buffer, 0.1 M sodium acetate, pH 4.0, and again the coupling buffer. Ten ml of the CE were added to the MAb-coupled resin and rotated for 2 h before packing into a PD-10 column (GE Healthcare). Native Fel d 1 was eluted from the column by using 0.1 M glycine-HCl, pH 2.5. The eluate was neutralized immediately with 1 M Tris-HCl, pH 8.0, dialyzed against PBS before concentrating to a desired protein concentration.

Titration for optimal concentrations of the MAb and PAb and other reagents and conditions for detecting Fel d 1

Titrations of MAb, PAb and goat anti-rabbit immunoglobulin-HRP conjugate and conditions of the sandwich ELISA were done (three times) according to the ELISA Guidebook.²⁹ Checkerboard titration was performed for determining the optimal concentrations of the mouse MAbC48 and the rabbit PAb for detecting fixed amount of Fel d 1. Purified MAbC48 IgG was diluted two-fold serially in 100 µl coating buffer ranging from 4.0 to 0.03 µg/100 µl and these were used to coat wells of an ELISA plate (Costar®, USA). After keeping the plate at 4°C for 16 h, all wells were washed with PBST and blocked with 300 µl of 2% BSA in PBST (blocking solution) at 25°C for 1 h. Excess blocking solution was removed by washing, then 100 ng of rFel d 1 in 100 µl of diluent (1% BSA in PBST) were added to the MAb-coated wells. The plate was kept at 37°C for 1 h. After washing as above, wells were added with 100 µl of ten-fold serially diluted rabbit PAb from 1:100 to 1:10,000 in diluent. The plate was kept at 37°C for 1 h, washed with PBST, and 100 µl of goat anti-rabbit immunoglobulin-horseradish peroxidase (HRP) conjugate (Southern Biotech, USA) at 1:2,000 (optimal and economic dilution from titration) were added to all wells. After incubating further at 37°C for 1 h and washing, ABTS substrate (KPL, USA) were added and the preparation was kept in darkness for 30 min. The enzymatic reaction was stopped by adding 100 µl of 1% SDS in diluent. OD_{405nm} of the content of all wells were determined against blank (wells to which PBS was added instead of the rFel d 1) by using an ELISA reader (Multiscan EX; LabSystem, Finland). Optimal concentrations of the mouse MAb and rabbit PAb were their lowest concentrations that yielded the highest ELISA signal against the rFel d 1.

Collection and extraction of house dust samples

Dust samples were collected from houses of 36 cat allergic subjects who were positive by skin prick test to the cat crude

hair extract. The dust sample collection received consensus from Siriraj International Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok (COA no. Si119/2016). For the sample collection, a dust collector was applied for two min on one square meter area of the floor in the patient's bedroom. One hundred mg of each sample was mixed with 1 ml of PBS and centrifuged at 5,000 x g, 25°C, 10 min. The supernatant was kept in small aliquots at -20°C until use.

Quantification of Fel d 1 in dust samples

A sandwich ELISA (MAbC48-allergen-rabbit PAb format) was used for quantification of Fel d 1 in the dust samples. ELISA wells were coated individually with optimal amount of purified MAbC48 IgG1 in 100 µl of coating buffer at 4°C, 16 h. After washing with PBST, the wells were blocked with 300 µl of 1% BSA in PBS. Various amounts of native Fel d 1 (standard allergen for constructing a standard curve) or samples were added in triplicate to the blocked wells, incubated at 37°C for 1 h, and washed. Each well was then added with rabbit PAb to rFel d 1 (optimal dilution from titration) in 100 µl PBST and kept further for 1 h. Goat anti-rabbit immunoglobulin-HRP conjugate (Southern Biotech; 1:2,000) and ABTS substrate (KPL, USA) were used for color development with appropriate incubation and washing between the steps. OD_{405nm} of the content in each well was determined against blank (BSA blocked wells added with PBS instead of the allergen sample). A standard curve was constructed from the OD_{405nm} of the known native Fel d 1 amounts and the Fel d 1 content of the sample was extrapolated from the standard curve. Negative ELISA was OD_{405nm} < 0.05.³⁰

Amounts of the Fel d 1 in the dust samples were also quantified by using commercially available test kit (INDOOR Biotechnologies, USA) which is a sandwich ELISA [MAb6F9-allergen sample-biotinylated-MAb3E4 format based on a universal allergen standard (UAS)].³¹ Streptavidin-HRP (Southern Biotech) and ABTS (KPL) substrate were used for color development. Fel d 1 content of each sample was calculated from the UAS standard curve.

Statistical analysis

Spearman's rank correlation was used for determining the correlation of the Fel d 1 amounts in the house dust samples quantified by using the locally made reagents and the commercial test kit.

Results

Average amounts of total proteins and native Fel d 1 extracted from 50 g of the cats' hair were 22.18 and 2.533 mg, respectively. **Figure 1A** shows proteins contained in the cat hair extract after SDS-PAGE and CBB staining. The proteins ranged in molecular masses from >10 to >170 kDa. The 6× histidine tagged-recombinant protein purified from the transformed *E. coli* lysate after SDS-PAGE and CBB staining is shown as a single band at ~23-24 kDa (**Figure 1B**). The protein was found to be rFel d 1 by mass spectrometric analysis (**Table 1**). From 50 g of dogs' hair, 13.72 mg of total proteins were obtained.

Two weeks after giving the last rFel d 1 immunizing dose, the rabbit was bled and the immune serum was collected. The indirect ELISA titer (calculated by EC50) of the rabbit

immune serum against the purified Fel d 1 was 1:64,000. At dilution 1:1,000, the PAb yielded OD_{405nm} at 2.319 and 0.102 against 0.1 µg of 6× His tagged-rFel d 1 and 6× His tagged-recombinant internalin A (control antigen), respectively. Both 6×-His tagged-proteins (0.1 µg) gave OD_{405nm} at 0.514 and 0.493 when they were detected by anti-6×-His antibody, respectively.

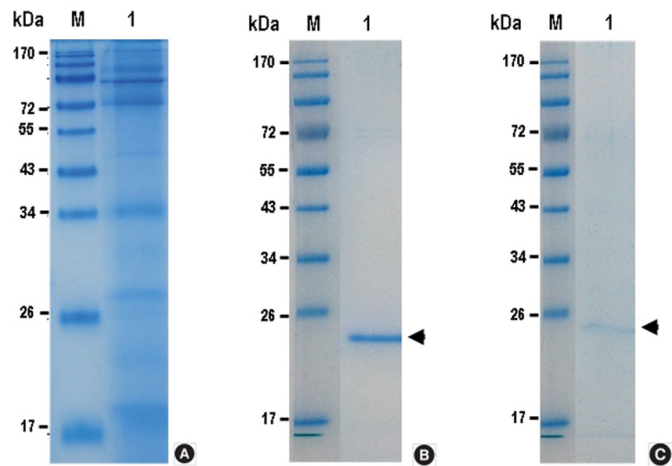


Figure 1. Protein profiles of crude cat hair extract (CE), purified recombinant Fel d 1, and purified native Fel d 1. Proteins contained in the crude cat hair extract (CE) after SDS-PAGE and Coomassie Brilliant Blue G-250 staining (lane 1 of A); purified recombinant Fel d 1 (lane 1 of B; arrow); and purified native Fel d 1 (lane 1 of C; arrow). M of all panels, protein standard markers. Numbers at the left of all panels are protein masses in kDa.

Table 1. LC-MS/MS Mascot results of in-gel tryptic digestion of recombinant protein thought to be rFel d 1 searching against Swiss-Prot database.

Protein	Orthologous protein	Accession no.	Peptide matches	Score	Matched peptide sequence(s) (score)
Recombinant protein thought to be rFel d 1	Major allergen I polypeptide chain 1 [<i>Felis catus</i>]	FEL1A_FELCA	4	473	ILKNCVDAK (72) ALPVLENAR (54) DVDLFLTGTPDEYVEQVAQYK (97) RDVDLFLTGTPDEYVEQVAQYK (63)

Table 2. Checkerboard titration of rabbit polyclonal IgG (PAb) and mouse MAb for detecting fixed amount of rFel d 1 by sandwich ELISA (representative of three independent experiments).

MAb (µg/well)	PAb dilution						Blank
	1:100	1:500	1:1,000	1:2,000	1:5,000	1:10,000	
4	1.124*	0.939	0.798	0.844	0.72	0.682	0
2	1.699	1.651	1.651	1.547	1.355	0.939	0
1	1.387	1.434	1.35	1.202	1.066	0.843	0
0.5	1.371	1.33	1.249	1.185	0.995	0.754	0
0.25	1.4	1.332	1.312	1.279	1.149	0.901	0
0.125	1.632	1.425	1.264	1.167	1.181	0.78	0
0.0625	1.481	1.313	1.289	1.139	1.056	0.724	0
0.03125	1.804	1.403	1.444	1.151	1.196	-0.035	0

* OD_{405nm} value that was read against blank (well to which PBS was added instead of the rFel d 1). In the sandwich ELISA for detecting Fel d 1 in dust samples, MAbC48 at 2 µg was used for coating an ELISA well and rabbit PAb at the dilution 1:1,000 was used.

The results indicate that the PAb contained principally anti-rFel d 1 and negligible amount of anti-6×-His. The IgG (PAb) prepared from the immune serum was adjusted to 1 mg/ml.

For preparing Fel d 1 specific MAb, the hybridoma clone C48 was grown in serum free medium to the maximal phase of growth. Culture supernatant of the hybridoma had the titer (calculated by EC50) at 1:16 to rFel d 1. The MAb IgG1 purified from the hybridoma culture supernatant by using HiTrap Protein-G HP column (GE Healthcare) was adjusted to 1 mg/ml of PBS.

Native Fel d 1 was isolated from the crude cat hair extract by using MAbC48-coated resin. **Figure 1C** shows SDS-PAGE-separated pattern of the nFel d 1 (25 kDa) purified from the CE by using MAbC48-affinity resin. The slightly higher molecular mass of the nFel d 1 than the rFel d 1 should be due to glycosylation of the former.

The results of the checkerboard titration of the mouse MAbC48 and the rabbit PAb are shown in **Table 2**. The optimal amount of the MAbC48 for coating each ELISA well was 2 µg and the optimal and economic dilution of the PAb was 1:1,000. The MAb concentration at 2 µg/well is rather high and we could use much less amount of the MAb to coat the ELISA well. However, in using less MAb, the amount of the PAb had to be increased. Because PAb obtained from animal immunization tends to have batch-to-batch and animal-to-animal variation, we chose to use more MAb than PAb as the cultured hybridoma secretes MAb with the same quality and specificity in all cultures. By using 2 µg of the MAb as a capture antibody and 1:1,000 rabbit PAb as a detection reagent, it was found that the lowest amount of the purified nFel d 1 that could be detected

by the sandwich ELISA was ~0.039 ng (Figure 2). The ELISA gave negligible signal when the IgG of non-immune serum of the same rabbit at various dilutions were used as the Fel d 1 detection reagent (data not shown).

The test kit did not give positive result to the heterologous antigens namely crude extracts of dogs' hair, *D. farinae*, *D. pteronyssinus*, and *P. americana*.

Amounts of Fel d 1 in the 36 dust samples determined by using the commercialized test kit in comparison with the sandwich ELISA using the locally made reagents are shown in Table 3. The levels of the Fel d 1 in dust samples detected by

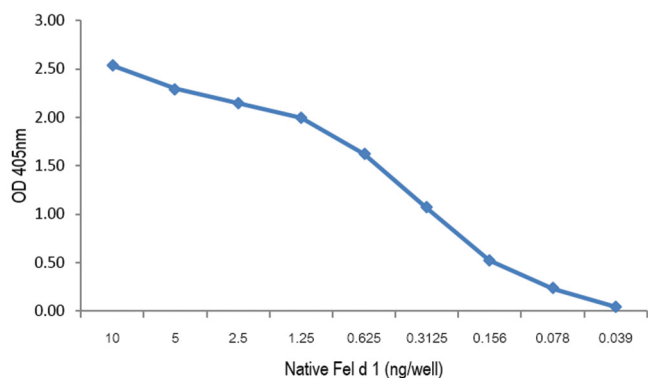


Figure 2. Lowest native Fel d 1 detection level of the sandwich ELISA using local reagents. Sandwich ELISA using 2 µg of MAbsC48 as the Fel d 1 capture reagent and 1:1,000 rabbit polyclonal IgG to recombinant Fel d 1 as the Fel d 1 detection reagent. Known amounts of nFel d 1, ranged from 10-0.039 ng/well were used in the assay. The lowest amount of the purified nFel d 1 that could be detected by the two-side ELISA was ~0.039 ng/well (OD_{405nm} > 0.05 which was used as a cut-off OD between positive and negative ELISA).

using the commercialized reagents ranged from undetectable (sample 30) to 11.313 µg per gram of dust (columns 3, 6 and 9 of Table 3). Fel d 1 detection by using the locally made reagents and native Fel d 1 as a standard ranged from undetectable (samples 25, 27, and 30) to 11.078 µg per gram of dust (columns 2, 5 and 8 of Table 3). Few measurements (samples 24, 26, 31, 34 and 35) showed large differences between both assays (i.e. up to 11.8-fold in sample 24). Nevertheless, a correlation coefficient (*r*) by Spearman's rank correlation of the two test kits (based on the Fel d 1 detectable 33 samples) was 0.923. Thus, there is a strong correlation of the two assays. Formerly, the Fel d 1 level higher than 1 µg per gram of dust (based on ELISA using Fel d 1 extract as standard) was regarded as the level for cat sensitization and symptoms and the level higher than 8 µg per gram of dust was the asthmatic aggravation level.³¹ However, recent investigation³¹ has indicated that a Fel d 1 quantitative difference of 3.4 fold lower was observed when the universal allergen standard (UAS) consisted of 8 purified natural allergens: Der p 1, Der f 1, Der p 2, Fel d 1, Can f 1, Rat n 1, Mus m 1, and Bla g 2, was used to construct a standard curve in comparison to the amounts obtained by using the individual extract based-ELISA. By using the correction factor of 3.4 of the UAS based-ELISA, the levels of cat sensitization and morbidity and asthmatic aggravation should become 0.29 and 2.35 µg per gram of dust, respectively. Among the 36 dust samples, there were 16 (nos. 5, 9, 17, 18, 19, 20, 21, 24, 26, 28, 29, and 32-36) and 10 samples (nos. 17, 19, 20, 21, 26, 28, 29, 32, 33, and 35) that contained sensitization levels of Fel d 1 as determined by the local and commercial test kits, respectively. Nevertheless, both test kits identified similarly 7 samples (nos. 20, 26, 28, 29, 32, 33 and 35) that contained asthmatic aggravation levels of the allergen.

Table 3. Amounts of Fel d 1 in dust samples collected from houses of 36 cat allergic patients as determined by the local and commercialized test kits.

Sample no.	Level of Fel d 1 (µg/g of dust) as detected by using		Sample no.	Level of Fel d 1 (µg/g of dust) as detected by using		Sample no.	Level of Fel d 1 (µg/g of dust) as detected by using	
	Local reagents	Commercial test kit based on UAS*		Local reagents	Commercial test kit based on UAS*		Local reagents	Commercial test kit based on UAS*
1	0.069	0.024	13	0.187	0.073	25	-	0.052
2	0.029	0.014	14	0.099	0.031	26	2.791	11.313
3	0.263	0.142	15	0.089	0.028	27	-	0.051
4	0.034	0.002	16	0.249	0.059	28	11.065	11.313
5	0.739	0.284	17	0.773	0.930	29	9.977	11.313
6	0.106	0.029	18	0.908	0.281	30	-	-
7	0.115	0.088	19	0.874	0.755	31	0.243	0.092
8	0.068	0.169	20	3.456	2.483	32	11.078	11.313
9	0.509	0.162	21	0.331	0.379	33	10.441	11.313
10	0.111	0.044	22	0.039	0.028	34	0.369	0.086
11	0.129	0.060	23	0.039	0.028	35	4.116	11.313
12	0.016	0.009	24	1.690	0.143	36	0.308	0.158

-, not detectable; *, Universal Allergen Standard (INDOOR Biotechnologies, USA)

Discussion

Fel d 1 is produced by squamous epithelial cells of the cat's lacrimal, salivary, and sebaceous glands. The allergen is predominant on the pet's skin and fur and also in anal sac.^{3,20,32-35} The amounts of cat-produced Fel d 1 varied depending on several factors including the sexes (hormones) and anatomical sites.^{24,36} Uncastrated males produce more Fel d 1 than the neutered ones and females; this is because testosterone increases the glandular secretions.^{24,36} In the cat skin, the mean Fel d 1 level was significantly higher on the animal facial area than chest.³⁴ The respective levels in the fur of these two locations were 63.6 ± 34 and 29.6 ± 13.6 $\mu\text{g/g}$ of hair, respectively.³⁴ Cat washing reduces significantly the allergen levels; but after 2 days the normal concentrations were restored.³⁴ Cat allergens are airborne and thus they are found ubiquitously in the environment. Non-cat keepers can be sensitized by the environmental cat allergens.

The immunoassay for detecting Fel d 1 in environmental samples has been developed since 1988¹ and quantification reagents are commercially available only from INDOOR Biotechnologies, USA. The commercialized test kit is a sandwich ELISA-based method using 8 purified native allergens (natural allergens: Der p 1, Der f 1, Der p 2, Fel d 1, Can f 1, Rat n 1, Mus m 1, and Bla g 2) as a standard (UAS).³¹ The mouse monoclonal antibody, MAb6F9, was used as the allergen capture reagent and biotin-labeled MAb3E4 was for Fel d 1 detection. Both MAbs bound specifically to different Fel d 1 epitopes. The MAb6F9 and MAb3E4 were derived from spleen of a mouse immunized with native Fel d 1. The Fel d 1 quantification test kit in this study is also a sandwich ELISA using mouse MAbC48 against rFel d 1 (specific to a novel IgE-binding conformational epitope of the Fel d 1)²¹ as a capture antibody and a rabbit polyclonal antibody to rFel d 1 as a detection reagent. Similar MAb-allergen-PAb format was used with success previously in the detection and quantification of cockroach allergens, i.e., Per a 1 and Per a 9.^{37,38} We found previously that the PAb-allergen-MAb format was less sensitive than the MAb-allergen-PAb format. For the former format, it was possible that non-specific components in the samples also bound to the PAb coated onto the ELISA well surface and, in the effect, made it difficult for the MAb which was added later to find the specific epitope, as there might be a steric hindrance effect by the non-specific epitopes/proteins present in the sample. Therefore, the more sensitive MAb-allergen-PAb format was used in this study. The test kit did not give positive result to crude extracts of dogs' hair, *D. farinae*, *D. pteronyssinus*, *P. americana*, and *B. germanica*, indicating specificity of the assay.

The locally made test kit using extracted and purified native Fel d 1 as standard was compared with the commercial test kit based on UAS for detection and quantification of Fel d 1 in 36 dust samples collected from houses of the cat allergic patients located in Bangkok metropolitan area. From the 36 samples, 3 samples (nos. 25, 27, and 30) were below the detection limit of the locally made test kit while 1 sample (no. 30) could not be detected by the commercial reagents indicating that the limit of detection of the locally made test kit is slightly lower than the commercially available test kit. This might be due to the fact that the PAb used as the Fel d 1 detection reagent of the locally made test kit contained also non-specific IgG. Purification of the

rabbit polyclonal antibodies by using recombinant/native Fel d 1 affinity resin should yield the more specific PAb and should increase the assay sensitivity. However, due to limitation of the allergens (native Fel d 1 extracted from the hair of healthy female cat that the owner brought to the clinic for spaying and purified bacterially-derived recombinant Fel d 1), we decided to use the PAb that were isolated and purified from the rabbit immune serum against rFel d 1 by means of ammonium sulfate precipitation and Protein-A affinity resin instead. Nevertheless, the data obtained using the locally made kit and the commercial test kit had a correlation coefficient (r) of 0.923. Formerly, Fel d 1 levels less than 1 $\mu\text{g/gram}$ of dust were unlikely to cause sensitization and allergic symptoms and 8 μg Fel d 1 per gram of dust was suggested as exposure threshold for asthma aggravation of cat sensitized subjects.³¹ By using a multi-allergen standard for calibration of the immunoassays, the novel Fel d 1 levels for cat sensitization and symptoms and asthmatic aggravation should be adjusted to 0.29 and 2.35 $\mu\text{g/gram}$ of dust respectively.³¹ There were 16 and 10 dust samples that contained the Fel d 1 above the sensitization threshold when detected by local and commercial reagents, respectively. Disagreement on the total number of samples that contained sensitizing Fel d 1 levels performed by the local and commercial tests (16 *versus* 10, respectively) could be due to different Fel d 1 epitopes recognized by the capture and detection reagents and different revelation systems of the two test kits. Other explanation for a large differences in absolute values measured by both assays for few samples (such as for samples 24, 26, 31, 34 and 35) could be due to differences in the sensitizing molecule used to develop the antibodies. The two mouse monoclonal antibodies in the commercial ELISA (MAb 6F9 and MAb 3E4) were raised against natural Fel d 1, whereas the mouse monoclonal and rabbit polyclonal antibodies used in the locally prepared assay as the Fel d 1 capture and detection reagents, respectively, were raised against recombinant rFel d 1. The rFel d 1 has the two subunits (chains 1 and 2) artificially linked and thus the heterodimers might assemble differently compared with the naturally occurring counterpart.^{6,39} Bond et al.³⁹ showed that recombinant Fel d 1 displayed IgE-binding activity and was able to induce histamine release, but at the lower level than the naturally occurring Fel d 1 heterodimers. Thus, differences in oligomerization between the recombinant and natural allergens could lead to differences in the measurements obtained with both assays. Nevertheless, both test kits showed identical results in identification of the samples that contained asthma aggravated levels of Fel d 1.

It is noteworthy that polymorphism of Fel d 1, especially the chain 2 exists due to variation in glycosylation and/or truncated forms.⁴⁻⁶ Salivary gland secretes long chain 2 of 92 amino acid residues while sebaceous gland produced short form of 90 residues.⁵ Most Fel d 1 specific monoclonal antibodies including our MAbC48 recognized epitopes in chain 1.^{1,21,40} Thus, the assays should not be affected by the chain 2 polymorphism. Two truncated Fel d 1 heterodimers of 13-14 and 16-17 kDa have been identified in four different anatomical sites of the cats including cheek zone, interdental zone, anal sac, and chest area.⁴¹ Therefore, it is important that the antibodies used in the Fel d 1 quantification should

be directed to the epitope(s) that are conserved for all variants of the Fel d 1.

In conclusion, a two-site (sandwich) ELISA for detection and quantification of Fel d 1 was invented using the MAb specific to a recently recognized Fel d 1 IgE-binding conformational epitope as the allergen capture reagent. Rabbit anti-recombinant Fel d 1 polyclonal IgG and the purified native Fel d 1 were used as the allergen detection reagent and the standard allergen, respectively. Performance of the locally made reagents was comparable to the commercially available reagents. There was a strong correlation ($r = 0.923$) of the results obtained by using the two test kits beyond chance. The locally invented sandwich ELISA is reliable for future use especially in a situation when the commercial test kit is not available or slow to come, or in developing localities where health facility resources are limited.

Acknowledgements

The work was co-supported by the National Research University (NRU) Project of the Office of Commission on Higher Education (CHE), Ministry of Education, RSA5780025 of the Thailand Research Fund (TRF), Faculty of Medicine Siriraj Hospital, Mahidol University and the NSTDA Chair Professor grant of the National Science and Technology Development Agency (NSTDA) funded by the Crown Property Bureau. Nitat Sookrung is a scholar of the RSA grant of the TRF and Natt Tassaniyananda received the NRU grant.

References

- Chapman MD, Aalberse RC, Brown MJ, Platts-Mills TA. Monoclonal antibodies to the major feline allergen Fel d 1. II. Single step affinity purification of Fel d 1, N-terminal sequence analysis, and development of a sensitive two-site immunoassay to assess Fel d 1 exposure. *J Immunol.* 1988;140:812-8.
- Phipatanakul W. Animal allergens and their control. *Curr Allergy Asthma Rep.* 2001;1:461-5.
- Dabrowski AJ, Van der Brempt X, Soler M, Seguret N, Lucciani P, Charpin D, et al. Cat skin as an important source of Fel d 1 allergen. *J Allergy Clin Immunol.* 1990;86:462-5.
- Morgenstern JP, Griffith IJ, Brauer AW, Rogers BL, Bond JF, Chapman MD, et al. Amino acid sequence of Fel d 1, the major allergen of the domestic cat: protein sequence analysis and cDNA cloning. *Proc Natl Acad Sci U S A.* 1991;88:9690-4.
- Griffith IJ, Craig S, Pollock J, Yu XB, Morgenstern JP, Rogers BL. Expression and genomic structure of the genes encoding FdI, the major allergen from the domestic cat. *Gene.* 1992;113:263-8.
- Kaiser L, Grönlund H, Sandalova T, Ljunggren HG, van Hage-Hamsten M, Achour A, et al. The crystal structure of the major cat allergen Fel d 1, a member of the secretoglobulin family. *J Biol Chem.* 2003;278:37730-5.
- Mukherjee AB, Zhang Z, Chilton BS. Uteroglobin: a steroid-inducible immunomodulatory protein that founded the Secretoglobulin superfamily. *Endocr Rev.* 2007;28:707-25.
- Spitzauer S, Pandjaitan B, Söregi G, Mühl S, Ebner C, Kraft D, et al. IgE cross-reactivities against albumins in patients allergic to animals. *J Allergy Clin Immunol.* 1995;96:951-9.
- van Ree R, van Leeuwen WA, Bulder I, Bond J, Aalberse RC. Purified natural and recombinant Fel d 1 and cat albumin in *in vitro* diagnostics for cat allergy. *J Allergy Clin Immunol.* 1999;104:1223-30.
- Cabañas R, López-Serrano MC, Carreira J, Ventas P, Polo F, Caballero MT, et al. Importance of albumin in cross-reactivity among cat, dog and horse allergens. *J Investig Allergol Clin Immunol.* 2000;10:71-7.
- Sastre J. Molecular diagnosis in allergy. *Clin Exp Allergy.* 2010;40:1442-60.
- Grundström J, Neimert-Andersson T, Kemi C, Nilsson OB, Saarne T, Andersson M, et al. Covalent coupling of vitamin D3 to the major cat allergen Fel d 1 improves the effects of allergen-specific immunotherapy in a mouse model for cat allergy. *Int Arch Allergy Immunol.* 2012;157:136-46.
- Smith W, Butler AJ, Hazell LA, Chapman MD, Pomés A, Nickels DG, et al. Fel d 4, a cat lipocalin allergen. *Clin Exp Allergy.* 2004;34:1732-8.
- Ichikawa K, Vailes LD, Pomés A, Chapman MD. Molecular cloning, expression and modelling of cat allergen, cystatin (Fel d 3), a cysteine protease inhibitor. *Clin Exp Allergy.* 2001;31:1279-86.
- Adédoyin J, Johansson SG, Grönlund H, van Hage M. Interference in immunoassays by human IgM with specificity for the carbohydrate moiety of animal proteins. *J Immunol Methods.* 2006;310:117-25.
- Adédoyin J, Grönlund H, Oman H, Johansson SG, van Hage M. Cat IgA, representative of new carbohydrate cross-reactive allergens. *J Allergy Clin Immunol.* 2007;119:640-5.
- Smith W, O'Neil SE, Hales BJ, Chai TL, Hazell LA, Tanyaratrisakul S, Piboonpocanum S, Thomas WR. Two newly identified cat allergens: the von Ebner gland protein Fel d 7 and the latherin-like protein Fel d 8. *Int Arch Allergy Immunol.* 2011;156:159-70.
- Grönlund H, Adédoyin J, Reininger R, Varga EM, Zach M, Fredriksson M, et al. Higher immunoglobulin E antibody levels to recombinant Fel d 1 in cat-allergic children with asthma compared with rhinoconjunctivitis. *Clin Exp Allergy.* 2008;38:1275-81.
- Kristensen AK, Schou C, Roepstorff P. Determination of isoforms, N-linked glycan structure and disulfide bond linkages of the major cat allergen Fel d 1 by a mass spectrometric approach. *Biol Chem.* 1997;378:899-908.
- van Milligen FJ, van 't Hof W, van den Berg M, Aalberse RC. IgE epitopes on the cat (*Felis domesticus*) major allergen Fel d 1: a study with overlapping synthetic peptides. *J Allergy Clin Immunol.* 1994;93:34-43.
- Tasaniyananda N, Tungtrongchitr A, Seesuy W, Sakolvaree Y, Indrawattana N, Chaicumpa W, et al. A novel IgE-binding epitope of cat major allergen, Fel d 1. *Biochem Biophys Res Commun.* 2016;470:593-8.
- Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Grönlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy.* 1999;29:896-904.
- Valenta R. Recombinant allergen-based concepts for diagnosis and therapy of type I allergy. *Allergy.* 2002;57:66-7.
- Treudler R, Simon JC. Overview of component resolved diagnostics. *Curr Allergy Asthma Rep.* 2013;13:110-7.
- Sookrung N, Chaicumpa W, Tungtrongchitr A, Vichyanond P, Bunnag C, Ramasoota P, et al. *Periplaneta americana* arginine kinase as a major cockroach allergen among Thai patients with major cockroach allergies. *Environ Health Perspect.* 2006;114:875-80.
- Sookrung N, Choopong J, Seesuy W, Indrawattana N, Chaicumpa W, Tungtrongchitr A. Allergenicity of native and recombinant major allergen groups 1 and 2 of *Dermatophagoides* mites in mite sensitive Thai patients. *Asian Pac J Allergy Immunol.* 2016;34:51-8.
- Choopong J, Reamtong O, Sookrung N, Seesuy W, Indrawattana N, Sakolvaree Y, et al. Proteome, Allergenome, and novel allergens of house dust mite, *Dermatophagoides farinae*. *J Proteome Res.* 2016;15:422-30.
- Rogers BL, Morgenstern JP, Garman RD, Bond JF, Kuo MC. Recombinant Fel d 1: Expression, purification, IgE binding and reaction with cat-allergic human T cells. *Mol Immunol.* 1993;30:559-68.
- Crowther JR. The ELISA Guidebook. Second Edition. Hamana Press. Springer Science+Business Media, LLC, New York, USA. 2009
- Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev.* 2008;29:S49-S52
- Filep S, Tsay A, Vailes L, Gadermaier G, Ferreira F, Matsui E, et al. A multi-allergen standard for the calibration of immunoassays: CREATE principles applied to eight purified allergens. *Allergy.* 2012;67:235-41.
- Woodfolk JA, Luczynska CM, de Blay F, Chapman MD, Platts-Mills TA. Cat allergy. *Ann Allergy.* 1992;69:273-5.
- Mata P, Charpin D, Charpin C, Lucciani P, Vervloet D. Fel d 1 allergen: skin and or saliva? *Ann Allergy.* 1992;69:321-2.
- De Andrade AD, Birnbaum J, Magalon C, Magnol JP, Lanteaume A, Charpin D, et al. Fel d 1 levels in cat anal glands. *Clin Exp Allergy.* 1996;26:178-80.
- Carayol N, Birnbaum J, Magnan A, Ramadour M, Lanteaume A, Vervloet D, et al. Fel d 1 production in the cat skin varies according to anatomical sites. *Allergy.* 2000;55:570-3.
- Ramadour M, Birnbaum J, Magalon C, Lanteaume A, Charpin D, Vervloet D. Cat sex differences in major allergen production (Fel d 1). *J Allergy Clin Immunol.* 1998;101:282-4.
- Sookrung N, Diraphat P, Chaicumpa W, Tongtawe P, Sakolvaree Y, Tapchaisri P, et al. Cockroach allergen detection and cockroach allergens of allergic Thai patients. *Asian Pac J Allergy Immunol.* 2003;21:1-9.

38. Tungtrongchitr A, Sookrung N, Munkong N, Mahakittikun V, Chinabut P, Chaicumpa W, et al. The levels of cockroach allergen in relation to cockroach species and allergic diseases in Thai patients. *Asian Pac J Allergy Immunol.* 2004;22:115-21.
 39. Bond JF, Brauer AW, Segal DB, Nault AK, Rogers BL, Kuo MC. Native and recombinant Fel d 1 as probes into the relationship of allergen structure to human IgE immunoreactivity. *Mol Immunol.* 1993;30:1529-41.
 40. Slunt JB, Rogers BL, Chapman MD. IgE antibodies to recombinant forms of Fel d 1: dichotomy between fluid-phase and solid-phase binding studies. *J Allergy Clin Immunol.* 1995;95:1221-8.
 41. Bienboire-Frosini C, Lebrun R, Vervloet D, Pageat P, Ronin C. Distribution of core fragments from the major cat allergen Fel d 1 is maintained among the main anatomical sites of production. *Int Arch Allergy Immunol.* 2010;152:197-206.
-