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Sero-prevalence study of IgE responses to allergens from Malaysian house dust (HDM) and storage mites (SM)

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Abstract. Allergens of *Dermatophagoides* and *Blomia* species are well-characterized but not for other species. This study was conducted to determine the prevalence of allergic sensitization to house dust (HDM) and storage mites (SM). One hundred adult subjects (aged ≥ 18) were recruited. The mite specific IgE of all allergic subjects were higher compared with healthy subjetcs despite being not statistically significant except for *D. farina*e and *G. malaysiensis*. The mean serum IgE levels against HDM and SM for allergic subjects were significantly higher compared with those in healthy subjects. They were mainly sensitized to *Dermatophagoides farinae* (35%) and *Glycycometus malaysiensis* (37%). Immunoblots revealed not all allergic subjects showed positive immuno-reactivity against the mites tested. Single or multiple bands were observed for different species. The subjects were commonly sensitized to Group 2 (9-12 kDa), 10 (38 kDa) and 18 (40-48 kDa) allergens. Twenty-one out of 60 allergic subjects were sensitized to either one or more species. The majority of them (71%) were sensitized to single species. The allergic subjects were mainly sensitized to *D. pteronyssinus*, followed by *Tyrophagus putrecentiae* and *Aleuroglyphus ovatus*. Seven were solely sensitized to HDM while 10 were solely sensitized to SM. Four subjects were sensitized to both. Pre-adsorption study revealed no cross-reactivity. There was difference between the prevalence and reactivity to allergens of HDM and SM in these subjects. Both ELISA and immunoblot did not correlate well but can complement each other in improving the detection of mite allergens to the species level.

INTRODUCTION

Mites are found globally and those which live together with human can be categorized into house dust mites (HDM) and storage mites (SM). The commonest HDM species are *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blomia tropicalis* and *Euroglyphus maynei* (Vrtala *et al.*, 2014). SM (flour, forage or grain mites) are normally present in storage facilities for grains and may grow in processed foods such as flour, cereals and baking mixes (Arlian & Morgan, 2003). Mites have been incriminated to trigger many allergic diseases. Zahedi *et al.* (2011) reported that 61% of 580 Malaysian children with allergic rhinitis had positive skin prick test to aeroallergens and HDM accounted for more than half of the sensitization: *D. pteronyssinus* (27.9%), *D. farinae* (26.4%) and *B. tropicalis* (26.0%). Similarly in Singapore, sensitization to *D. pteronyssinus* (89%), *B. tropicalis* (70%), or both mites (91%) were reported in 253 children with allergic rhinitis, atopic dermatitis or asthma (Kidon *et al.*, 2011). The most prevalent IgE responses in these children were against Der p 1 (64%), Der p 2 (71%), Blo t 5 (45%), Blo t 7 (44%), and Blo t 21 (56%).

To date, 33 groups of mite allergens have been identified and characterized (http://www.allergen.org). Groups 1 and 2 allergens are the most common allergens to which

the majority of mite-allergic patients are sensitized to. However, the allergens or allergenicity of other species especially *A. ovatus* and *G. malaysiensis*, which are prevalent in Malaysian houses, are not adequately studied. Thus, detailed characterization and understanding of the similarities, differences and cross-reactivities among different mite species, and other allergens are necessary for accurate diagnosis and effective allergen targeted immunotherapy. This study aims to determine the prevalence and nature of sensitization to HDM and SM in an urban-based private hospital in Penang, to measure the level of mite-specific serum IgE using ELISA, and to identify distinct allergens responsible for the symptoms in those patients.

MATERIALS AND METHODS

Culture of mites and preparation of mite crude extracts

Three HDM (*D. pteronyssinus*, *D. farinae* and *B. tropicalis*) and three SM species (*G. malaysiensis*, *A. ovatus* and *T. putrescentiae*) were maintained in fish flakes (TetraMin Crisps, Germany) at 25ºC and 75% relative humidity inside culture flasks (Nunc, Denmark).

The cultures were checked regularly under a stereomicroscope (Nikon, Japan) for growth and any contamination. The confluent mite cultures were harvested using floatation method with saturated sodium chloride (NaCl) solution. The mites and faecal pellets were collected and homogenized using Microsmash homogenizer (Tomy, Japan) in the presence of 500 µL phosphate buffered saline (PBS) and glass beads (1.0 mm diameter; Tomy, Japan). The suspension was homogenized at 5,000 rpm for 30 seconds followed by cooling at -80ºC for 5 minutes before the next homogenization cycle. This process was repeated for 15 cycles until ~80% of the mites were fragmented. The homogenate was collected, centrifuged at 8,000 rpm for 10 minutes at 4ºC. Supernatant was collected, quantified using Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, USA).

Subject Recruitment and Collection of Blood Samples

This study was approved by the Research and Ethical Committee of International Medical University. Subjects (aged ≥ 18) who attended Loh Guan Lye Specialist Centre, a major 300-bed private hospital in Penang for their health screening were invited. Written consent was obtained from each subject. Standard clinico-demographic details and blood samples were collected from 60 allergic and 40 healthy subjects. Allergic subjects were defined as those who had a physician-diagonsed asthma and/or allergic rhinitis. Healthy subjects were those without serious medical illness and allergic conditions as certified by health screening doctors. Any subjects who were on chronic anti-histamine or corticosteroids therapy and/or with chronic diseases such as chronic liver or renal diseases were excluded. The allergic subjects were recruited from the hospital chest and allergic clinics. The healthy subjects were recruited from subjects who attended the hospital for general healthcare screening.

Enzyme-linked immunosorbent assay

Mite-specific IgE was quantified using ELISA with the respective crude mite extracts. Mite extracts (50 µL of 0.1 µg/µL per well) in carbonate coating buffer (pH 9.6) were coated on Immulon-2^{HB} Removawell strips (Thermo Scientific, USA) overnight at 4ºC. The strips were washed thrice with PBS (pH 7.4) on the following day and were blocked with 1% BSA (Sigma-Aldrich Corporation, USA) for an hour at room temperature (RT). The strips were then washed thrice with PBS and were incubated with sera (1: 50 in PBS; 50 µL per well) for two hours at 37ºC. PBS was included as negative control. The strips were then washed thrice with PBS-Tween, followed by incubation with 50 µL each of horseradish peroxidase conjugated goat anti-human IgE (1: 2000; Kirkegaard & Perry Laboratories, Maryland) for an hour at RT. Upon completion of incubation, the strips were washed thrice with PBS. SureBlue™ TMB Microwell peroxidase substrate (100 µL each; Kirkegaard & Perry Laboratories) was added and incubated for 15 minutes in the dark. Eventually, the reaction was stopped after 15

minutes by adding 50 µL each of 2.5 M sulphuric acid. Absorbance was measured using a microplate reader (Dynex Opsys, USA) at 450 nm with 630 nm as a reference filter. A subject is considered to be sensitized to HDM or SM when the mite-specific IgE level is above the cut-off values as determined (cut-off value = $mean_{negative}$ + 2 standard deviation).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE (12% resolving gel and 4% stacking gel) was used to separate mite extracts. Equal volume of mite extract (15 µL) was mixed with Laemmli sample buffer and β-mercaptoethanol (Bio-Rad Laboratories, USA), heated at 90ºC for 10 minutes and cooled on ice. The mite extract was separated using Mini-PROTEAN tetra cell (Bio-Rad Laboratories) at 110 V for 90 minutes. Upon completion, the gels were removed and rinsed thoroughly with excessive ultrapure water. One gel was stained with SimplyBlue™ SafeStain Coomassie® G-250 (Invitrogen, USA).

The other gel with 2-wells (one for protein markers and one for 200 µL of mite extract mixed with Laemmli sample buffer and β-mercaptoethanol) was rinsed with excessive volume of ultrapure water and the proteins were transferred onto a nitrocellulose membrane (0.45 µm; Bio-Rad Laboratories) using mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories) at 80 V for 70 minutes. Once the transfer was completed, the membrane was removed and rinsed with excess ultrapure water. A small portion of the membrane was cut and stained with Ponceau S (Sigma-Aldrich Inc., USA) for transfer efficiency check. The remaining portion of the membrane was blocked with 5% skim milk in Tris buffered saline (TBS)-Tween for 45 minutes on a shaker. The membrane was rinsed with ultrapure water and was cut into strips. Pre-absorption study was performed by incubating equal volumes of 1 mg/mL crude protein extract and the patient serum (1: 50) at 4ºC overnight with gentle shaking. The extract-antibody mixtures were centrifuged at 5,000 rpm for 10 minutes in order to remove the immune complexes and any precipitate. Each nitrocellulose membrane strip was incubated with subject's serum (800 µL; 1: 50, diluted with 5% skim milk in TBS-Tween) or the supernatant collected after pre-adsorption study (800 µL) overnight at 4ºC on a shaker. On the following day, the strips were rinsed with ultrapure water and washed with TBS-Tween thrice for 10 minutes each on a shaker (150 rpm). The strips were then incubated with horseradish peroxidase conjugated goat anti-human IgE (800 µL; 1: 500; KPL) for 30 minutes on a shaker at RT. Next, the strips were rinsed with ultrapure water and washed thrice with TBS-Tween for 10 minutes each. Finally, 4-chloro-1-naphthol (Sigma-Aldrich Inc., USA) was added for the colour development of the immune-reactive bands. The relative migration distances of the markers and the immune-reactive bands were measured and their MW were determined.

Statistical analysis

Student's *t* test was performed with SPSS version 18 for Windows (SPSS Incorporation, Chicago, Illinois) to determine the statistical differences between healthy and allergic subjects. P values were considered statistically significant if they were less than 0.05.

RESULTS

The demographic profiles of all subjects are summarized in Table 1a. The nature and severity of the allergic subjects against four well-known mites are summarized in Table 2. Overall, the mite-specific IgE levels of all allergic subjects were higher compared with healthy subjects for all the six species studied but did not achieve statistical significant except for *D. farina*e and *G. malaysiensis* (Table 1b). The means IgE levels against HDM (0.159) and SM (0.129) for allergic subjects were significantly higher ($P < 0.01$) compared with the means (0.087 and 0.069 respectively) of the healthy subjects. The majority of the subjects were sensitized to *D. farinae* (35/100) and *G. malaysiensis*

Table 1. (a) Demographic profiles of recruited subjects. (b) Mite-specific IgE levels in healthy and allergic subjects. (c) Subjects with IgE levels above cut-off values

(a)		Healthy*	Allergic**
Total, n		**40**	**60**
Male, n (%)		17 (42.5)	23 (38.3)
Female, n (%)		23 (57.5)	37 (61.7)
Age, years (Mean ± SD)		29.2 (± 9.7)	29.6 (± 11.7)
Race, n			
Chinese, n (%)		29 (72.5)	40 (66.7)
Malay, n (%)		8 (20.0)	12 (20.0)
Indian, n (%)		3 (7.5)	5 (8.3)
Others, n (%)		0 (0)	3 (5.0)
Occupation	**Total**		
Student, n	**53**	19	33
Health Care, n	**23**	11	12
Education, n	**6**	4	2
Admin, n	**7**	4	3
Designer, n	**1**	1	0
Other, n	**11**	6	5

(b)	HDM			SM		
	Dp	**Df**	**Bt**	**Gm**	**Ao**	**Tp**
Mean healthy, OD (± SD)	0.084 (± 0.096)	0.098 (± 0.047)	0.079 (± 0.055)	0.088 (± 0.040)	0.048 (± 0.032)	0.071 (± 0.048)
Mean allergic, OD (± SD)	0.133 (± 0.255)	0.224 (± 0.325)	0.119 (± 0.145)	0.203 (± 0.173)	0.075 (± 0.101)	0.110 (± 0.136)
P values	0.244	0.004	0.056	0.000	0.059	0.082

(c)	HDM			SM		
Cut-off values	**Dp 0.309**	**Df 0.154**	**Bt 0.352**	**Gm 0.142**	**Ao 0.194**	**Tp 0.412**
Total, n	**6**	**35**	**5**	**37**	**4**	**2**
Healthy, n (%)	1 (16.67)	9 (25.71)	0 (0)	14 (37.84)	1 (25.00)	0 (0)
Allergic, n (%)	5 (83.33)	26 (74.29)	5 (100.00)	23 (62.16)	3 (75.00)	2 (100.00)
P values	0.865	0.313	0	0.259	0.643	0
Genders						
Male, n (%)	4 (66.67)	17 (48.57)	3 (60.00)	15 (40.54)	3 (75.00)	2 (100.0)
Female, n (%)	2 (33.33)	18 (51.43)	2 (40.00)	22 (59.46)	1 (25.00)	0 (0)
Races						
Chinese, n (%)	4 (66.67)	22 (62.86)	4 (80.00)	24 (64.86)	1 (25.00)	1 (50.00)
Malay, n (%)	2 (33.33)	9 (25.71)	1 (20.00)	10 (27.03)	3 (75.00)	1 (50.00)
Indian, n (%)	0 (0)	3 (8.57)	0(0)	3 (8.11)	0 (0)	0 (0)
Others, n (%)	0 (0)	1 (2.86)	0(0)	0 (0)	0 (0)	0(0)

* Normal or healthy subjects age ≥ 18 years with no asthma and allergic rhinitis, chronic (liver and renal) diseases and not taking anti-histamine / corticosteroid medication.
** Patient age ≥ 18 with either asthma or allergic rhinitis or both.
HDM: house dust mites; SM: storage mites; Tp: *Tyrophagus putrescentiae*; Dp: *Dermatophagoides pteronyssinus*; Df: *Dermatophagoides farinae*; Bt: *Blomia tropicalis*; Ao: *Aleuroglyphus ovatus*; Gm: *Austroglycyphagus/Glycycometus malaysiensis.*

Table 2. Skin prick test results of the recruited allergic subjects to four known dust mite (*D. farinae*, *D. pteronyssinus*, *B. tropicalis* and *T. putrecentiae*) allergens (Soluprick, ALK-Abello, United Kingdom). Each value is expressed as diameter of wheal size in mililitres (mm). Note: The values are in cm of longest diameter of the wheal of skin prick test. A positive reaction to an allergen is defined as a wheal ≥ 3mm, or measuring ≥ to that of histamine wheal. The test is invalid if the saline control produces a wheal

Patient No.*	Histamine	Saline	*Dermatophagoides farinae*	*Dermatophagoides pteronyssinus*	*Blomia tropicalis*	*Tyrophagus putrescentiae*
1	0.5	0	1.0	0.7	0.4	0.3
3	0.7	0	3.1	2.7	3.0	1.0
4	0.4	0	1.3	1.4	1.4	0.4
6	0.5	0	1.0	1.5	1.4	1.4
7	0.4	0	0.8	1.3	1.0	1.1
8	1.3	0	1.5	1.0	1.1	1.0
9	0.5	0	2.0	1.4	0.4	0.5
10	0.6	0	0.8	0.7	0.6	0.9
11	0.4	0	0	0	0	0
12	0.6	0	0.6	0.7	0.7	0.3
13	0.5	0	0.4	0.3	0.4	0.5
14	0.6	0	2.2	1.4	0.8	0.8
15	0.5	0	0.6	0.6	0.8	0.5
16	0.6	0	1.5	1.3	0.5	0.5
17	0.4	0	1.1	0.8	0.5	0.6
18	0.6	0	0.9	1.8	1.1	0.6
19	0.4	0	0.5	0.6	1.4	0.3
20	0.5	0	0.8	0.7	0.8	0.9
21	0.5	0	2.0	1.9	1.8	0.5
22	0.3	0	0.6	0.7	0.5	0.9
24	0.6	0	1.4	0.8	0.4	0.8
25	0.3	0	1.2	1.5	1.1	0.6
26	0.5	0	1.5.	1.3	1.6	1.0
27	0.5	0	0.7	0.6	2.2	0.5
28	0.5	0	1.7	0.7	1.8	0.4
29	0.7	0	1.3	1.3	0.8	0.8
30	0.6	0	0.2	0.1	2.5	1
31	0.7	0	1.0	0.8	1.5	1.2
32	0.6	0	1.6	1.1	0.9	0.6
33	0.9	0	1.3	0.7	0.9	0.8
34	0.9	0	0.2	0.2	0.2	0
35	0.4	0	0.8	1.2	0.8	0.8
37	0.4	0	0.8	0.9	1.2	1.8
38	0.6	0	0.9	0.7	0.8	0.7
39	0.6	0	1.0	1.0	0.8	0.6
40	0.6	0	1.5	1.5	1.3	1.8
41	0.4	0	0.3	0.5	0.3	0.3
42	0.5	0	0.5	0.3	0.3	0.4
43	0.8	0	1.3	1.3	1.0	1.1
44	0.4	0	0.9	1.6	0.5	0.6
46	0.1	0	0	0	0	0.2
47	0.5	0	0.6	0.3	0.3	0.5
48	0.6	0	1.3	0.7	0.8	1.2
49	0.3	0	0.6	0.4	0.3	0.7
50	0.5	0	0.6	0.3	1.0	0.6
53	0.5	0	0.8	1.0	0.7	0.6
57	0.4	0	0.8	0.9	1.8	0.7
58	0.5	0	0.3	0	0	0.3
60	0.7	0	0.4	0	1.1	0.5
62	0.5	0	0.3	0.2	0	0
63	0.4	0	1.2	1.1	0.8	0.3
71	0.5	0	0	0	0	0
73	0.5	0	1.4	1.8	1.4	1.2
77	0.6	0	1.0	1.6	1.1	0.5
80	0.6	0	0	0	0	0
92	0.4	0	0.5	1.3	0.5	0.6
93	0.9	0	0.3	0	0	0
94	0.6	0	0	0	0	0
95	0.3	0	0.9	0.6	0.7	0.6
96	0.6	0	1.7	2.0	1.7	1.6

(37/100) (Table 1c). Less than 10 subjects had high levels of mite-specific IgE against *D. pteronyssinus* (6/100), *B. tropicalis* (5/100), *A. ovatus* (4/100) and *T. putrescentiae* (2/100). However, there were several healthy subjects with high levels of mite-specific IgE against *G. malaysiensis* and *D. farinae*.

IgE reactivity to *G. malaysiensis* showed a strong significant correlation with reactivity to *D. farinae* (Spearman's rho correlation coefficient = 0.73). There are significant weak correlations of IgE reactivity among the other species tested.

Immunoblot analysis revealed fourteen subjects (14%) were reactived against *D. pteronyssinus* (Fig. 1). Among them, five were healthy adults without asthma, allergic rhinitis, and chronic (liver and renal) diseases. Subject No. 95, an allergic subject, had the highest number (seven) of prominent immune-reactive bands ranging from 11 to 108 kDa. Five subjects (35.71%) showed a similar band at 38 kDa which was probably a Group 10 allergen based on the published data on allergen grouping. The other deduced common allergens were Groups 4 (35.71%) and 18 allergens (42.86%). However, five healthy subjects (Nos. 67, 69, 87, 105 and 116) exhibited immuno-reactive peptides of 45-51 kDa; subjects Nos. 105 and 116 reacted with peptides of 38 kDa (Fig. 1b).

Unexpectedly, only one allergic subject (No. 95) showed positive reactivity against *D. farinae* (Fig. 2). Two intense and prominent bands were observed at approximately 10 (possible Group 2 allergen) and 131 kDa (not an identified allergen as yet). Smearing was also noticed throughout the membrane.

Although *B. tropicalis* is a common HDM found in subtropical and tropical regions, only 4 allergic subjects showed positive immuno-reactivity against it (Fig. 2). Only faint bands were observed at 8-33 kDa. Subject No. 95 had smearing at the region of high MW. The same subject had ~20 kDa band as subject No. 6 and that peptide is hypothesized as Group 3 allergen based on calculated MW.

A total of six subjects, of whom 2 were healthy adults, showed positive immune-reactivity against *G. malaysiensis* (Fig. 3). Allergic subjects were immuno-reactive against *G. malaysiensis* allergen of ~10 kDa (Group 2 allergen) except for subject No. 48 at 60 kDa. One of the healthy subjects also exhibited similar band at 10 kDa while another non-allergic subject had immuno-reactivity against allergen of 31 kDa (Group 10).

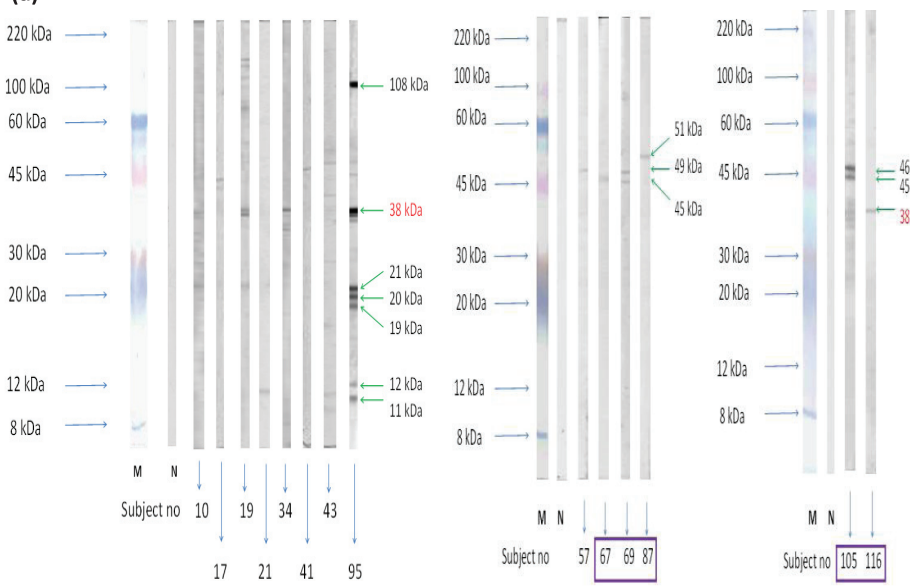
Although only 6 subjects had high mite-specific IgE levels against *A. ovatus*, 14 subjects were found to have immuno-reactive bands in which 6 out of 14 were healthy subjects (Fig. 4). Two allergic subjects (Subject Nos. 95 and 19) shared multiple prominent IgE-binding bands above 37 kDa. The rest of the subjects showed only 1 or 2 immuno-reactive bands ranging from 27 to 60 kDa. Similarly, 4 subjects showed immuno-reactivity against Group 10 allergen (32-38 kDa). Reactivity to Group 4 allergens (~55 kDa) was commonly observed among both allergic and healthy subjects.

Eight allergic and one healthy subjects were immuno-reactive against *T. putrescentiae* allergens (Fig. 5). Two allergic subjects (Nos. 19 and 95) shared relatively similar immuno-reactivity patterns against this mite. The majority of these subjects exhibited one or two immuno-reactive bands against *T. putrescentiae* of < 60 kDa (except for subjects Nos. 26 and 75). The 38 kDa allergen appeared to be the common immuno-reactive band for this species among these subjects.

Overall, 21 out of 60 allergic subjects recruited in the present study were sensitized either to one or more mite species tested. The majority of them (71%) were sensitized to a single mite species. The allergic subjects were mainly sensitized to *D. pteronyssinus*, followed by *T. putrecentiae* and *A. ovatus*. Subject No. 95 was sensitized to all the six species studied. Subject No. 19 was sensitized to four species (*D. pteronyssinus*, *B. tropicalis*, *A. ovatus* and *T. putrecentiae*). Only one out of 100 subjects was sensitized to *D. farinae*. Out of 21 sensitized subjects, only 7 were solely sensitized to HDM (*D. pteronyssinus*, *D. farinae* and *B. tropicalis*) while 10 were solely sensitized to storage mites (*A. ovatus*, *G. malaysiensis* and *T. putrecentiae*). Four subjects were sensitized to both HDM and SM.

Thirteen out of 40 healthy subjects were sensitized to one mite species except for

(a)

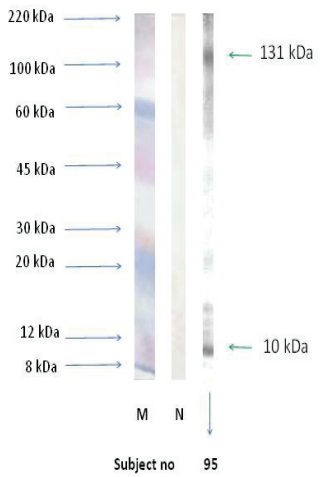


(b)

Subject No	Migration Distance (mm)	Relative MW	Possible Allergen Group
10	24.5	38	Group 10
	33.5	21	Group 3
17	21	50	Group 4
	22	48	Group 18
19	5	134	
	6	126	
	8.5	108	
	11.5	90	
	24.5	38	Group 10
	25	37	Group 10
	34.5	22	Group 3
21	47.5	11	Group 2
34	24.5	38	Group 10
	27	35	Group 10
41	19.5	55	Group 4
43	19	57	Group 4
	23	45	Group 18
57	19	49	Group 18
67	20	45	Group 18
69	19.5	49	Group 18
	20.5	45	Group 18
87	17	51	Group 4
95	8.5	108	
	18	61	Group 15
	20	54	Group 4
	24.5	38	Group 10
	25	37	Group 10
	25.5	37	Group 10
	34	21	Group 3
	35	20	Group 3
	36.5	19	Group 3
	46	12	Group 2
	48	11	Group 2
105	19	46	Group 18
	20	45	Group 18
116	24	38	Group 10

Fig. 1. (a) Immunoblot analysis against *D. pteronyssinus*. Note: Numbers indicated allergic subjects with positive immune-reactivity; numbers within a box indicated healthy subjects with positive immune-reactivity; **N** indicated subject with no immune-reactivity; **M:** protein markers. (b) Relative molecular weights of immuno-reactive bands against *D. pteronyssinus*.

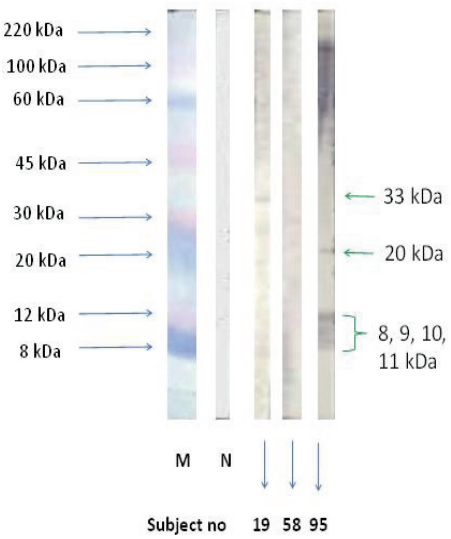
(a)

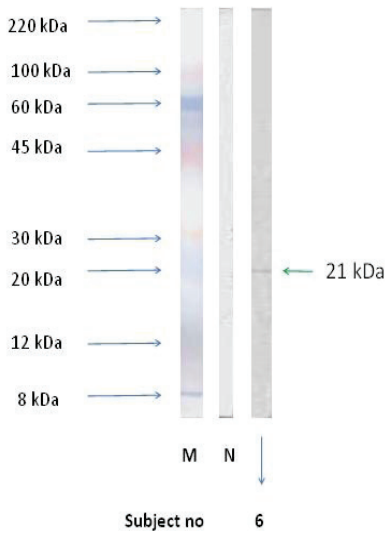


(b)

Subject No	Migration Distance (mm)	Relative MW	Possible Allergen Group
95	1	182	
	7	131	
	12	99	
	17	76	
	39	23	Group 1/3
	41	20	Group 3
	48.5	13	Group 2
	53	10	Group 2
	55	9	Group 2

(c)



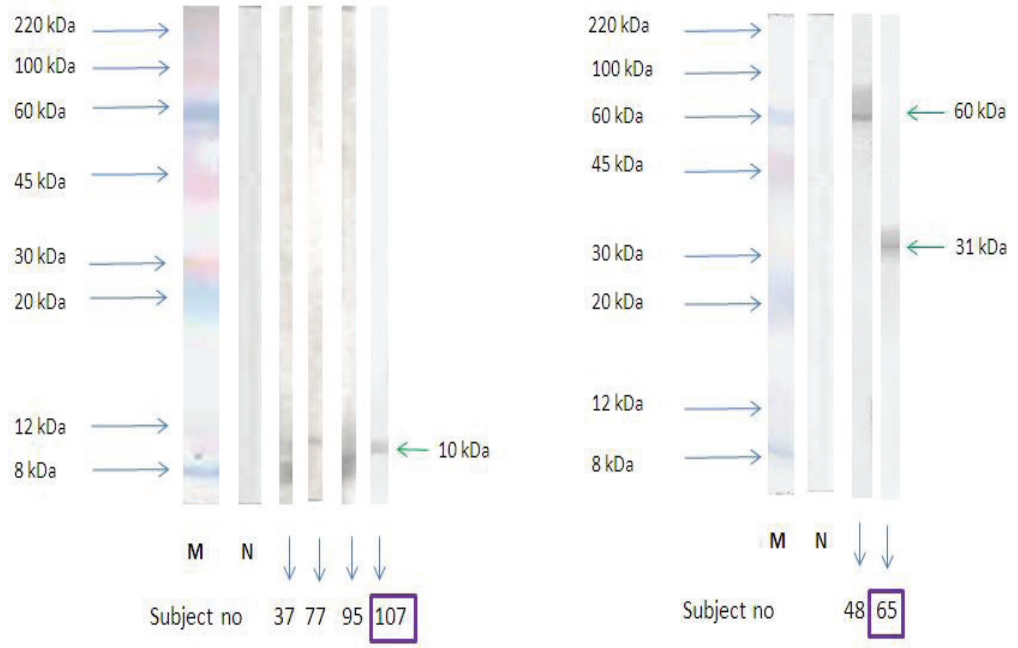


(d)

Subject No	Migration Distance (mm)	Relative MW	Possible Allergen Group
6	36.5	21	Group 3
19	25.5	33	Group 10
58	29	26	Group 1
95	33	20	Group 3
	42	11	Group 2
	44	10	Group 2
	45.5	9	Group 2
	46.5	8	Group 2

Fig. 2. Immunoblot analysis against (a) *D. farinae* and (c) *B. tropicalis*. Note: Numbers indicated allergic subjects with positive immune-reactivity; **N** indicated subject with no immune-reactivity; **M:** protein markers. Relative molecular weights of immuno-reactive bands against (b) *D. farinae* and (d) *B. tropicalis*.

(a)

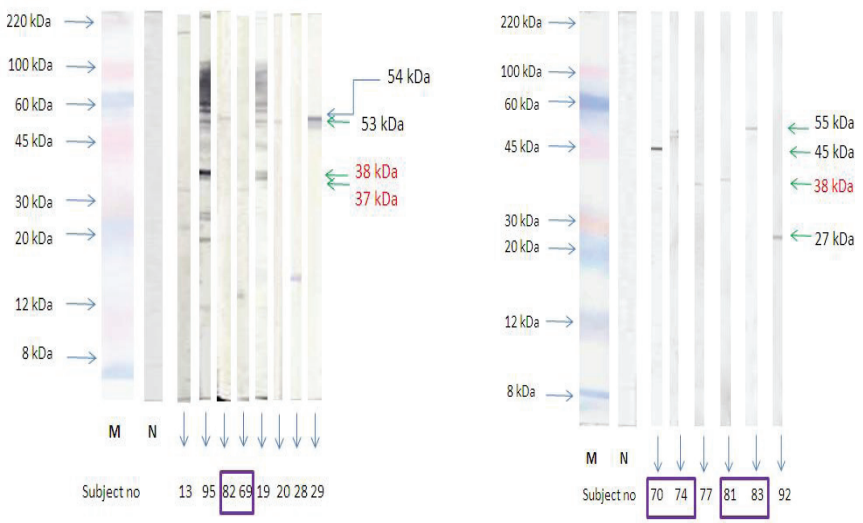


(b)

Subject No	Migration Distance (mm)	Relative MW	Possible Allergen Group
37	50	10	Group 2
	53	9	
48	12	60	Group 15
65	26	31	Group 10
77	50	10	Group 2
95	46	13	Group 2
	50	10	Group 2
	53	9	Group 2
107	51.5	10	Group 2

Fig. 3. (a) Immunoblot analysis against *G. malaysiensis*. Note: Numbers indicated allergic subjects with positive immune-reactivity; numbers within a box indicated healthy subjects with positive immune-reactivity; **N** indicated subject with no immune-reactivity; **M:** protein markers. (b) Relative molecular weights of immuno-reactive bands against *G. malaysiensis*.

(a)

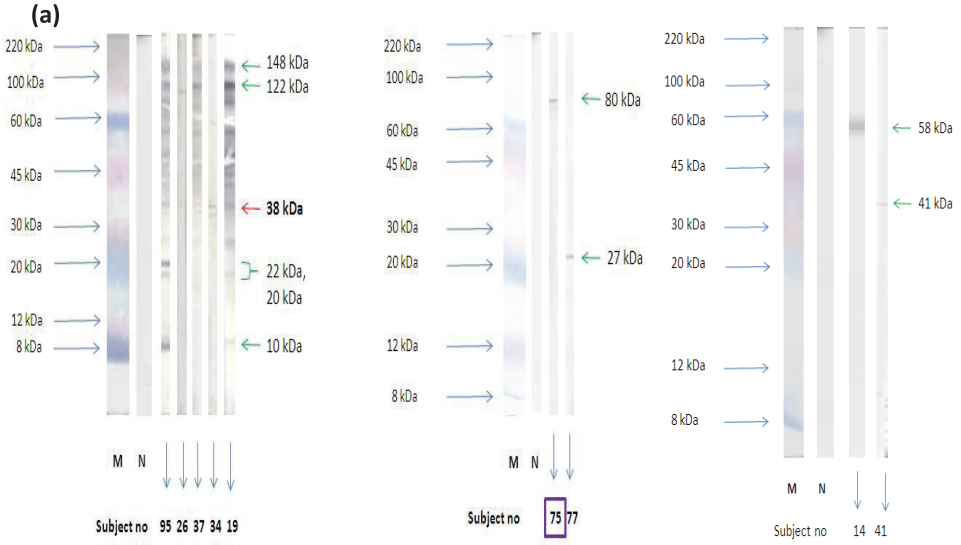


(b)

Subject No	Migration Distance (mm)	Relative MW	Possible Allergen Group
13	27	32	Group 10
	32	23	Group 1/3
95	2	169	
	14.5	60	Group 15
	15.5	54	Group 4
	17	53	Group 4
	24.5	38	Group 10
	25	37	Group 10
	30	27	Group 1/3
	31	25	Group 1/3
	34	20	
	44	11	Group 2
82	16	67	Group 15
69	18.5	57	Group 4
	26.5	34	Group 10
19	2	169	
	14.5	60	Group 15
	15.5	54	Group 4
	17	53	Group 4
	24.5	38	Group 10
	25	37	Group 10
20	16	54	Group 4
28	38.5	15	
	39.5	14	
29	16	54	Group 4
	17	53	Group 4
70	18	45	Group 18
74	16.5	55	Group 4
	17.5	54	Group 4
77	25	40	Group 18
81	24	43	Group 18
83	17	55	Group 4
92	32	27	Group 1/3

Fig. 4. (a) Immunoblot analysis against *A. ovatus*. Note: Numbers indicated allergic subjects with positive immune-reactivity; numbers within a box indicated healthy subjects with positive immune-reactivity; **N** indicated subject with no immune-reactivity; **M:** protein markers. (b) Relative molecular weights of immuno-reactive bands against *A. ovatus*.

(a)



(b)

Subject No	Migration Distance (mm)	Relative MW	Possible Allergen Group
95	5	148	
	8	122	
	10	107	
	12	94	
	15	77	
	18	64	Group 15
	23	46	Group 18
	26	38	Group 10
	34.5	22	Group 3
	36	20	Group 3
	46	10	Group 2
	47	10	Group 2
26	9	114	
	26	38	Group 10
37	5	148	
	8	122	
	15	64	Group 15
34	26	38	Group 10
	27.5	34	Group 10
19	5	148	
	8	122	
	10	107	
	15	77	
	18.5	62	Group 15
	21.5	51	Group 4
	23	46	Group 18
	26	38	Group 10
	32	26	Group 1
	36	20	Group 3
	46	10	Group 2
	47	10	Group 2
75	10	80	
77	33	27	Group 1
14	12	58	Group 4
41	21	41	Group 18

Fig. 5. (a) Immunoblot analysis against *T. putrescentiae*. Note: Numbers indicated allergic subjects with positive immune-reactivity; **N** indicated subject with no immune-reactivity; **M:** protein markers. (b) Relative molecular weights of immuno-reactive bands against *T. putrescentiae*.

subject No. 69 who was sensitized to two mite species. These subjects were mainly sensitized to either *D. pteronyssinus* or *A. ovatus*. Subject No. 69 was sensitized to *D. pteronyssinus* (45-49 kDa) and *A. ovatus* (34 and 57 kDa). Only four subjects were sensitized to HDM only whereas eight subjects were sensitized to SM only; as stated above, subject No. 69 was sensitized to both HDM (*D. pteronyssinus*) and SM (*A. ovatus*).

When Western blotting and ELISA profiles of the subjects were compared, the results did not correlate well (data not shown). Those with positive immuno-reactivity did not have increased levels of mite-specific serum IgE, and vice versa. All the healthy subjects with positive immuno-reactivity against the respective mite species tested were without increased levels of mite-specific IgE. Exact matching (100%) between Western blotting and ELISA results were observed only for two subjects (Nos. 19 and 95).

DISCUSSION

To date, the prevalence of mite infestation and geographic distribution of mites in houses in Malaysia especially Penang is still lacking. The majority of the populations were sensitized to *D. pteronyssinus* and *D. farinae* (Zahedi *et al.*, 2011; Thomas *et al.*, 2002). *D. pteronyssinus* and *B. tropicalis* were the commost HDM found in house dust in Klang Valley, Malaysia with a significant but much lesser contribution from *D. farinae* (Mariana *et al.*, 2000). These houses were infested with at least 6 mite species including *Cheyletus malaccensis*, *B. tropicalis*, *D. farinae*, *D. pteronyssinus*, *Malayoglyphus intermedius* and *Sturnophagoides brassiliensis*. However, the present ELISA findings revealed a different pattern of sensitization whereby majority of the subjects were sensitized to *D. farinae* (35%) and *G. malaysiensis* (37%), instead of *D. pteronyssinus*. These could be result from the limitation of using crude extracts rather than purified recombinant allergens. Despite of this, *D. farinae* was reported as the main source of mite allergens that cause sensitization in Korea (Son *et al.*, 2014). Only 6% of the subjects had increased mite-specific serum IgE levels against *B. tropicalis* despite it being the most abundant mite found in Malaysia and Singapore recently (Thomas, 2010). In this study, the subjects (70% Chinese) were recruited due to its convenient accessibility and proximity to the researchers. Hence due to this limitation, the findings of this study cannot be generalized to the whole population of Malaysia.

In adition, these descrepencies could be due to geographical variations that might have affected the distribution and growth of mite species (Thomas, 2010) as George Town is located on an island. The climatic condition [e.g. temperature, relative humidity (RH), salinity, thermal stability, breezes etc.] near the coastline may differ from inland areas. The coastal areas had been reported to have higher number of mites (especially for *D. pteronyssinus* and *D. farinae*), compared with the inland areas (Pagan *et al.*, 2011). *D. pteronyssinus* was reported to be more abundant in the coastal areas while *D. farinae* predominated in the inland areas (Macan *et al.*, 2003). Sensitization rates to mites were higher along the coastline (83%) and were 50% lower at regions of 5-30 km away from the coast inland (Moral *et al.*, 2008). Areas with low RH (< 50%) or drier climates had low mite infestations (Arlian *et al.*, 2002). *D. farina*e and *D. pteronyssinus* population densities decreased when temperature and RH were maintained at 21–22ºC and ≤ 50% respectively (Arlian *et al.*, 1998). The growth of *D. farinae* can be prevented by maintaining the RH below 35% for at least 22 hours per day even though the remainder of the day was maintained at 75–85% RH (Arlian *et al.*, 1999). This will eventually affect the density of mites at the studied sites.

Der p 1 levels were highest in the coastal area compared with the inland rural and urban areas (Macan *et al.*, 2003). Der f 1 levels, on the other hand, were highest in the inland urban area compared with inland rural and coastal areas. Hence, urbanization in George Town where the subjects were recruited may contribute to the differences in temperature between urban and rural areas due to accumulation of heat, CO_2 emission

and decrease green vegetation fraction (Kataokaa *et al.*, 2009). The mite growth rates were proven to be affected by change of surrounding temperature (Aspaly *et al.*, 2007). The lowest development threshold for *Acarus siro*, *A. ovatus* and *T. putrecentiae* was 10.2°C while the most optimum temperature was at 25°C. *T. putrecentiae* had the highest tolerable temperature threshold at 49°C, followed by *A. ovatus* and *A. siro* both at 38°C.

Yeoh *et al.* (2003) reported sensitization profiles of *D. pteronyssinus* and *B. tropicalis* among Singaporean and Malaysian subjects. The rhinitis subjects (124) were sensitized to Blo t extract (73%), Blo t 5 (50%), Der p extract (49%), Der p 1 (43%) and Der p 2 (36%) respectively. Similar to the present study, the non-rhinitis (105 healthy) subjects were also sensitized to Blo t extract (57%), Blo t 5 (23%), Der p extract (36%), Der p 1 (13%) and Der p 2 (16%) respectively. Recently, Zahedi *et al.* (2011) reported 61% of 580 Malaysian children with allergic rhinitis were sensitized to *D. pteronyssinus*, *D. farinae* and *B. tropicalis* at 28%, 26% and 26% respectively. In the neighboring country, Thailand, both the atopic children (62.5% of 40 subjects) and adults (51% of 45 subjects) were sensitized to *D. pteronyssinus* (Trakultivakorn & Nuglor, 2002). Sensitization to *B. tropicalis* was reported in 37.5% and 40% of the atopic children and adults respectively.

Dermatophagoides pteronyssinus and *B. tropicalis* were found prevalent in Malaysia (Nadchatram, 2005) but did not account for triggering the allergic reactions of the recruited subjects. This could be due to recent climatic change (global warming) even in tropical regions which may influence the growth rates and fecundity of the mites. The mean monthly rainfall and temperature significantly affected the growth rates of the mites. Seasonal studies conducted in India revealed that atmospheric temperature, RH and rainfall from June to August were the most optimum conditions for the growth of the mites (Sharma *et al.*, 2011). *Blomia* species have maximum growth rates during the month of July, followed by June and March. *Dermatophagoides* species prefers July and March. The least favorable season for the mites was reported from November to February.

In the present study, 37% of the subjects were sensitized to *G. malaysiensis*. Nadchatram (2005) reported similar findings where approximately 55.6% of rhinitis patients were sensitized to *G. malaysiensis* in Malaysia with 18.1% demonstrating strong reactions. The majority of the subjects sensitized to this species showed immuno-reactive bands at 9-10 kDa region. No major allergens have been reported for this species so far, hence further characterization of this allergen would be beneficial for the understanding of the allergenicity of this species in Malaysian population. Further study on this species is recommended due to its prevalence in storage facilities for grains (Nadchatram, 2005), and its association with allergic reactions.

Less than 5% of the subjects had increased levels of mite-specific serum IgE against *A. ovatus* and *T. putrescentiae*. They are SM and were associated with occupational environment such as farms, barns and storage facilities, and caused occupational allergic diseases (Arlian & Morgan, 2003; Thomas, 2010). The subjects were recruited from urban areas and the majority was students and healthcare professionals, hence limited exposure to these species is probably the reason for low sensitization rates in these subjects. Although both mites appeared to be less commonly involved in sensitization among the study population, nevertheless attention should be given as they can trigger serious medical emergency including oral mite anaphylaxis or food-induced anaphylaxis and other acariasis (Cui, 2014).

Aleuroglyphus ovatus is the only medically important SM of its genus. It is commonly found in stored products including grain products, seeds, dried fruits and dried anchovies (Thomas, 2010) and was first reported in West Malaysia in 1980 (Yunus & Ho, 1980). However, there are limited studies on this mite despite its increased association with anaphylaxis cases. *A. ovatus* thrives well within a wide range of temperatures, 20–32°C (average temperature in Malaysia is 27°C). It has a relatively shorter life cycle

compared with other species. These characteristics enable them to flourish in high numbers easily in various facilities and locations where food sources are available (Xia *et al*., 2009). Approximately 80% of individuals with respiratory allergy in Columbia were sensitized to *A. ovatus* (Putera *et al*., 1993). Contact dermatitis due to this species was reported in Australia (Geary *et al*., 2000). Sensitization to *A. ovatus* was reported in Florida (Nelson *et al*., 1996).

Immuno-blotting analysis of *D. pteronyssinus* revealed multiple immuno-reactive bands ranging from 11 to 130 kDa. Based on published data on relative MW, the deduced grouping for these allergens may be of Groups 2, 3, 4, 10 and 18. Even though the majority of the subjects had increased levels of serum IgE against *D. farinae*, only one subject's serum sample had several immuno-reactive bands ranging from 9-182 kDa. Thus, this subject may be sensitized to Group 2 or 3 allergens. The sensitization profiles of the studied subjects differ from those reported previously (Lind & Lowenstein, 1983; Macan *et al*., 2003; Moral *et al*., 2008) but the identity of the allergens involved needs to be confirmed.

As for *G. malaysiensis*, the majority of the subjects had immuno-reactivity against 9-15 kDa allergen. No established allergens have been reported so far for this species (http://www.allergen.org). Further study or characterization of this allergen is necessary since this species is commonly found in house dust here.

Overall, the majority of the subjects in the present study were sensitized to 38 kDa molecule which may correspond to Group 10 allergen. Group 10 allergen is a tropomyosin which is well-recognized for its cross-reactivity with tropomyosin from insects, mites, crustaceans and nematodes (Colloff, 2009) or other allergens. It is possible that these subjects were exposed to crustaceans like crabs and prawns, other insects or arthropods also.

Out of expectation, the findings obtained using ELISA and immunoblot differ. No correlation between the mite-specific serum IgE levels and positive immuno-reactivity was observed. The ELISA is an immunoassay with high sensitivity for the measurement of soluble antigen/antibody reaction. The most optimum condition for immunodiagnosis is by having specific and sensitive immuno-assays that are able to distinguish sensitized from those non-sensitized subjects and to have good correlation or confirmative diagnosis among the different methods used. However, this may not be feasible. The present discrepancy between ELISA and immunoblot analysis may be due to the denaturation of the mite allergens and the usage of crude extracts. Denaturation will lead to change in structural conformation which will eventually affected the binding affinity and avidity of the allergen to its antibody. Besides, a denatured allergen could expose more antigenic epitopes subsequent to unfolding and linearization for better recognition by IgE. A non-denaturing gel could be used in future studies to verify this hypothesis.

In order to improve the sensitivity and specificity of the ELISA, purified mite extracts or recombinant mite allergen could be used as this would minimize the non-specific interference and cross-reactivity by other impurities that are present in the crude mite extracts. However, the advantages and disadvantages of using recombinant allergens need to be carefully considered as they may not represent the native allergens that people are actually in contact with due to different conformational changes between native and recombinant allergens. Currently, commercially available ELISA kits are available for the detection of Der p 1, Der p 2, Der f 1, Der f 2 and Blo t 5 from Indoor Biotechnologics Inc. (United States).

The prevalence of mites in Penang should be studied in order to identify the species and level of mite infestation, and to compare the nature of sensitization with other regions in Malaysia and other countries. More subjects should be recruited for better statistical representation. Further characterization of the shared allergens (e.g. 38 kDa) and unique allergens using mass spectrometry will be beneficial for the understanding of sensitization. More attention should be paid on *A. ovatus* and *G. malaysiensis* in the future as the present

study showed high sensitization rates to these species.

In conclusion, there was a difference between the prevalence and reactivity to allergens of HDM and SM in the recruited subjects. All the allergic subjects had higher mite specific IgE levels against all the six species studied compared with the non-allergic subjects. This study revealed that the subjects were postulated to be sensitized to 3 common allergens: Groups 10 (38 kDa), 2 (9-12 kDa) and 18 (40-48 kDa) allergens. Immunoblot analysis revealed that majority of subjects was sensitized to single mite species while some showed sensitization to several species. There was no correlation between the mite-specific serum IgE levels and positive immuno-reactivity. Immuno-blotting analysis revealed different patterns of sensitization compared with ELISA. Each subject with positive immuno-reactivity exhibited unique distinctive patterns of sensitization against different species of mites. Only a few subjects shared similar IgE-binding bands. The 38 kDa allergen was commonly shared among the subjects. Hence, development of immunoassays for the detection of mite sensitization should not be based solely on the commonest allergen of the commonest mite species such as *Dermatophagoides* species only. Two or more immunoassays could be used to complement each other for confirmation of sensitization. The improvement in detection and understanding of the nature of sensitization will contribute significantly to the development of detection kits and immunotherapy.

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