Comparative identification of protein profiles and major allergens of saliva, salivary gland and whole body extracts of mosquito species in Thailand

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Summary

Allergic reactions to mosquito bites, such as generalized urticaria or severe local reactions are common problems worldwide. The diverse sources of allergen prepared from different mosquito body parts usage are a major obstacle to obtaining safe and effective tests and immunotherapy for mosquito bite allergy. Thus, the reactions are often not recognized and allergen immunotherapy is seldom used for severe reaction to mosquito bites. In a search for appropriate allergen sources, the protein profiles of saliva, salivary glands and whole body extracts were comparatively analyzed from 4 common mosquito species of Thailand and/or South East Asia; viz. *Culex quinquefasciatus, Aedes aegypti, Aedes albopictus* and a zoophilic strain, *Anopheles minimus*. The major allergens in the extracts which elicited specific IgE responses in the pooled sera of subjects allergic to mosquito bites were identified. It was concluded that mosquito saliva was the best source of allergens. Additionally, both species-specific and species-shared allergens of the 4 mosquito species were identified. The major saliva allergens having MWs of 36, 32 and 22 kDa were identified. The identificstion of major allergens should facilitate the production of specific recombinant allergens and contribute to improvement in the diagnosis and specific immunotherapy of Thai mosquito bite allergy patients. *(Asian Pac J Allergy Immunol 2010;28:162-9)*

Key words: *saliva, salivary gland, mosquito allergens, mosquito bite allergy*

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Introduction

Concomitant with a documented rise in global temperatures there has been an increase in tropical storms, cyclones and hurricanes which have exacerbated outbreaks of diseases spread by mosquito vectors throughout most parts of the world but specifically in South East Asia (SEA) and Micronesia.[1] Worldwide, there are over 3000 different species grouped into more than 40 genera.[2,3] In Thailand alone, the number of verified species of mosquito fauna increased dramatically to 436 species in 2005.[4]

The saliva of mosquitoes contains a number of pharmacologically active compounds inhibiting the body's protective innate immune responses, causing anticoagulation, impairing platelet formation, vasodilation and anti-inflammatory activities. Additionally, the saliva facilitates bacterial or parasitic transmission, initial colonization and allergens that induce allergic reactions[5, 6]

Mosquito bites can elicit both immediate as well as delayed hypersensitivity reactions[7,8] which

can result in cutaneous symptoms varying from small papules to large pruritic swellings.[3] The resulting allergy can be determined by a history of allergic reactions that develops following witnessed mosquito bites. However, most bites are painless and not directly observed, especially in young children.[9] In response to exposure to bites from mosquitoes, subjects with mosquito bite allergy produce serum specific IgE against allergens from the mosquito. Allergens which induce the specific IgE response have been identified leading to the development of immunoassays for an in vitro diagnosis of mosquito bite allergy.[10]

Antihistamines or prednisone are effective in reducing itching or severe large local reactions to mosquito bites. Nevertheless, it is highly recommended that individuals who are at risk for anaphylaxis (a severe IgE mediated hypersensitivity reaction that is rapid in onset which may cause respiratory compromise resulting in death) from mosquito bites, carry an epinephrine auto-injector whenever they are likely to be exposed to mosquitoes.[11,12] Immunotherapy with injections of gradually increasing doses of mosquito allergens has been shown to prevent reactions to mosquito bites.[3,13] Until recently, diverse sources of antigens prepared from different mosquito body parts, were used in the diagnosis and immunotherapy of mosquito bite allergy. However, considerable variations in the biological activity of these allergens have been reported.[14]

Several studies have focused on the immunogenic and allergenic activities of mosquito salivary components. The SDS-PAGE and immunoblot techniques provide an opportunity for analyzing multiple allergens or for studying the IgE responses to each and every allergen.[15,16] In *Aedes aegypti*, at least 8 proteins from the saliva have been identified as allergens which bind to the IgE of individuals living in Manitoba, Canada. More than 16 allergens have been described in the saliva of *Aedes albopictus*.[3] Seventeen allergens from *Ae. vexans* have also been recognized.[15] In salivary gland extracts, at least 19 allergens have been revealed.[17] Saliva from *Anopheles stephensi* has been shown to contain a high molecular weight glycoprotein endowed with intense neutrophil chemotactic activity which contributes to the inflammatory reaction through the accumulation of neutrophils at the site of the mosquito bite.[18]

Although different extracts have been analyzed for their protein and allergen content in western allergy patients, the same has not been done for the sera of mosquito bite allergy patients in Thailand. Moreover, there has been no comparison of the 3 sources of extracts in the same study. Therefore, it was the purpose of the present study to compare protein profiles and potential allergens derived from saliva, salivary glad extracts (SGE) and whole body extracts (WBE) for four of common mosquitoes in Thailand; i.e. *Culex quinquefasciatus, Aedes aegypti, Aedes albopictus* and *Anopheles minimus*. Additionally, a comparison was made between the species-specific and species-shared allergens in the three extract proteins across all four mosquito species. This is the first time such comparisons have been made and the results will contribute to an improvement of diagnosis and immunotherapeutic treatment of Thai mosquito bite allergy patients.

Methods

Study Subjects

This project was approved by the ethical committee for research involving human subjects, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. All patients gave written informed consent after they had received detailed information about the study. This cross-sectional study was performed by recruiting patients from the outpatient Departments of Dermatology and Pediatrics, Siriraj Hospital, Mahidol University in Bangkok, Thailand. Demographic data, consisting of a complete family history of atopy, the onset and time course of personal atopy and mosquito allergy were recorded. A thorough physical examination was carried out and the morphology and distribution of any skin lesions noted. Serum were obtained from 20 adults and children (age range between 1.5-68 years) with a history of mosquito bite allergies and the presence of skin lesions. These were classified into immediate reactions (N = 8), immediate & delayed reactions (N = 3), and delayed reactions (N = 9). A wheal or flare lesion occurring within several minutes and peaking at twenty minutes after an exposure indicated an immediate reaction. An indurated pruritic papule developing within a few hours, peaking at twenty-four to thirty-six

hours and diminishing over several days or weeks indicated a delayed response.[19]

Twenty healthy non-allergic subjects (age range between 18-40 years, males = 8, females = 12) without a history of allergic diseases whose serum total IgE levels were within the normal range at the time of blood collection served as controls. Pooled serum obtained from both patients and control subjects was used in this study.

Mosquitoes

Four species of adult female mosquitos were used in this study, i.e. *Culex quinquefasciatus*, *Aedes aegypti*, *Aedes albopictus*, and *Anopheles minimus*. They were maintained in the insectary in the Department of Entomology, Armed Forces Research Institute of Medical Sciences and the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University. The ambient room temperature was maintained at a constant 25-28 ° C, with a relative humidity of 80-90%, and under a 12/12 hours light/dark photo-period.

Mosquito Saliva, salivary Gland and Whole Body Extracts

Mosquito salivae were collected from *Cx. quinquefasciatus, Ae. aegypti, Ae. albopictus,* and *An. minimus* following the method of Boorman with some modifications[20] Briefly, living female mosquitoes were anesthetized with ether and after stabilizing the legs and wings, each proboscis was inserted into a capillary tube filled with 20 µl of distilled water. Salivation was stimulated by the application 0.5% (v/v) malathion in acetone to the thorax. After approximately one hr, the contents of the capillary tubes were collected, pooled and lyophilized. Mosquito salivary gland extract (SGE) was obtained from adult female mosquitoes that were also anesthetized with ether. Their salivary glands were dissected in 0.02 M phosphate-buffered saline (PBS), pH 7.2, under a stereomicroscope, and sonicated. A total of over 100 pairs of salivary glands were collected. Mosquito whole body extract (WBE) was prepared by crushing and grinding whole body parts in cold PBS and centrifuged at 8820 g for 30 min. The supernatant was collected and served as WBE in the subsequent procedures. The Bradford method[21] was used to determine the protein concentration of all extracts. The extracts were stored at −40°C until further use.

Characterization of Protein profiles from Mosquito Saliva, Salivary Gland and Whole Body Extracts

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed according to the method described by Laemmli[22] with some modifications. The protein components of each extract were separated according to their molecular weight. A 12% separating and a 5% stacking gel were used. The SDS-PAGE was carried out using a mini-PROTEIN® II dual slab cell apparatus with a model 3000 XI power supply, 200/240 VAC (Bio-Rad Laboratories, USA). To perform the electrophoresis, saliva, salivary gland and whole body extracts with a concentration of 5 µg / lane (from titration) were mixed with an equal volume of 2 × sample buffer. The mixture was boiled for 5 min, and then was loaded into each well of the stacking gel (20 µl/well). For each run, 5 µl of broad range standard molecular weight markers were positioned into the reference well. Electrophoresis was carried out using a constant voltage setting at 150 volts for approximately 60 min or when the tracking dye reached the bottom of the separating gel. After electrophoresis, protein bands of the separated antigen were detected by staining the gel with 0.2% Coomassie Brilliant Blue R stain. The molecular weight of the SDS-PAGE separated-antigens were determined by comparing the relative electrophoretic mobility of any unknown component with standard protein markers of known molecular weights (MW) run concurrently on the same slab gel. A linear relationship was obtained by plotting the relative mobility of the protein markers against the logarithmic values of their MW. Pre-stained SDS-PAGE broad range standards (Bio-Rad, USA) were used.

Detection of Major Allergens by an Enhanced Chemiluminescence Immunoblot Analysis

An enhanced chemiluminescence immunoblot was performed by transblotting the SDS-PAGE-separated proteins from the gels to a nitrocellulose membrane (NC) following the method of Towbin.[23] The transfer cell was connected to a power supply, model 200/2.0, constant voltage 220/240 V, 50/60 Hz (Bio-Rad Laboratories, USA). A current of 0.25 A/cm^2 at 100 volts was applied. Prior to performing the immuno-reaction, the unoccupied sites on the NC membrane were blocked with a solution of Tris-Buffered saline

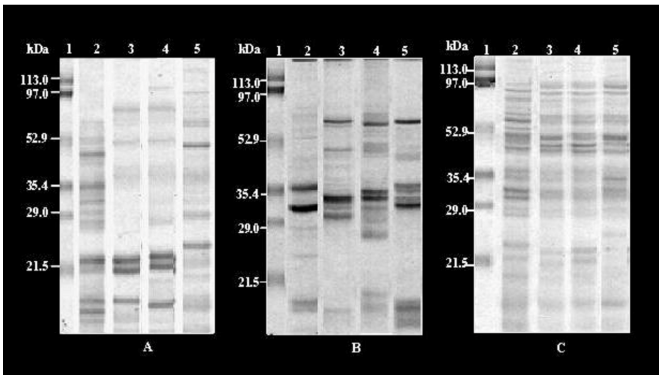

Figure 1. Protein profile patterns of saliva (panel A), salivary gland extracts (panel B) and the whole body extracts (panel C) of the four mosquito species stained with Coomassie Blue where lane 1 = molecular weight markers, lane 2 = *Cx. quinquefasciatus*, lane 3 = *Ae. aegypti,* lane 4 = *Ae. albopictuus* and lane 5 = *An. minimus*

Tween (TBS-T) with 5% non-fat dry powdered milk (w/v) at room temperature for 1 hour. After washing thoroughly, the NC was treated with the pooled patient's sera at the dilution of 1:2 and incubated at room temperature for 1 hour. Negative and positive controls were also included. Then, the NC was washed as in the previous step and probed with 1:1000 dilution of goat anti-human IgE-horseradish peroxidase conjugate (Dakopatte, Denmark). The reaction was processed at room temperature for 1 hour. After a thorough washing with TBS, the developing solution (ECL substrates) was prepared by mixing equal parts of the stable peroxide solution with the luminol solution (5 ml of each). The washed NC was incubated with the developing solution, swirling constantly. The NC was rinsed with TBS and then put face down on saran wrap, sealed and placed into an X-ray film cassette. The developed NC was exposed using Kodak X-Omat film (Eastman Kodak Co. Rochester, NY).

Total IgE Antibodies Determination

The total IgE levels in the sera of the control subjects were determined using a commercial kit (VIDAS, Biomerieux). All the assay steps were performed automatically and completed within approximately 30 minutes.

The results were automatically calculated using stored standard values and expressed in kilo international units/liter (KIU/I) standardized according to WHO's Second International Reference Preparation for human serum IgE, # 75/502. If the resultant values were < 150 KIU/L, the samples were classified as belonging to a non-atopic population, and therefore, considered as an indication of normalcy.

Statistical Analysis

Because the total number of detected proteins and allergens constituted measurement in an ordinal scale, the chi square test was used to compare the combined totals for each of the 4 mosquito species. The significance level of all inferential comparisons was $P \leq 0.05$.

Results

Saliva, Salivary gland and whole body proteins of Cx. quinquefasciatus, Ae. aegypti, Ae. albopictus and An. minimus

SDS-PAGE was carried out to profile the proteins content of saliva, SGE and WBE of the 4 mosquito species. Coomassie Brilliant Blue stained gel showed numerous bands ranging from 12.7 kDa to > 100 kDa. Figure 1 shows the outcome of protein analysis of the 4 species of mosquitoes in lanes 2 through 5 for *Cx. quinquefasciatus, Ae. aegypti, Ae. albopictus and*

Table 1. MW (kDas) of protein profiles in saliva of 4 mosquito species of Thailand characterized by SDS-PAGE

Protein (kDa)			
Cx. quinquefasciatus	*Ae. aegypti*	*Ae. albopictus*	*An. Minimus*
-	-	-	121.3
-	-	97.0	-
92.3	-	-	92.3
-	81.0	81.0	81.0
75.4	75.4	75.4	-
68.9	68.9	-	68.9
64.1	64.1	64.1	64.1
52.9	-	52.9	-
51.0	-	-	-
49.5	49.5	-	49.5
47.0	-	-	-
44.2	-	-	-
-	40.6	40.6	40.6
-	37.3	37.3	-
36.0	-	-	36.0
-	33.7	-	33.7
32.0	-	-	32.0
29.9	-	29.9	29.0
27.7	-	27.7	-
-	-	-	24.7
22.8	22.8	22.8	-
22.0	22.0	22.0	22.0
-	-	20.0	20.0
17.5	17.5	-	17.5
-	-	16.8	16.8
16.3	-	-	16.3
14.6	-	14.6	14.6
13.4	13.4	13.4	13.4

Table 2. MW (kDa) of protein profiles in salivary glands of 4 mosquito species of Thailand characterized by SDS-PAGE

Protein (kDa)			
Cx. quinquefasciatus	*Ae. aegypti*	*Ae. albopictus*	*An. Minimus*
112.0	-	-	-
-	-	103.0	-
95.0	-	-	-
-	88.5	88.5	-
-	-	81.3	-
74.1	74.1	74.1	74.1
-	-	72.2	-
-	70.1	70.1	70.1
-	-	68.6	-
-	67.3	67.3	-
-	66.0	66.0	-
63.5	-	-	63.5
-	-	60.1	-
57.6	-	57.6	57.6
56.7	-	56.7	-
-	-	-	54.6
52.2	-	52.2	-
-	-	50.9	50.9
-	49.5	49.5	49.5
-	48.0	48.0	-
-	47.4	47.4	47.4
46.1	-	46.1	46.1
43.8	-	-	-
-	-	39.8	39.8
37.5	37.5	37.5	37.5
36.7	36.7	36.7	36.7
35.5	35.5	35.5	35.5
-	34.2	-	34.2
-	33.5	33.5	33.5

Table 3. MW (kDa) of protein profiles in whole body extract of 4 mosquito species of Thailand characterized by SDS-PAGE

Protein (kDa)			
Cx. quinquefasciatus	*Ae. aegypti*	*Ae. albopictus*	*An. Minimus*
122.0	-	-	122.0
-	-	-	113.5
-	-	109.5	-
-	105.5	-	-
-	-	-	101.0
-	-	-	-
93.0	93.0	93.0	93.0
87.6	-	-	-
-	-	85.7	85.7
-	84.6	-	-
81.2	-	-	-
-	78.2	78.2	78.2
-	-	-	75.1
71.7	-	-	-
65.7	-	-	-
-	62.3	62.3	62.3
59.3	59.3	59.3	59.3
49.1	49.1	49.1	49.1
46.7	46.7	46.7	46.7
-	44.2	44.2	44.2
-	42.9	-	-
37.2	-	37.2	37.2
34.9	34.9	34.9	34.9
-	34.0	-	34.0
31.8	31.8	31.8	31.8
30.4	30.4	30.4	30.4
28.1	28.1	28.1	28.1
27.3	27.3	-	27.3
-	-	-	25.0

An. Minimus, respectively. The molecular weights of the salivaary proteins of the 4 mosquito species are presented in Table 1. The molecular weights of the proteins from the salivary gland extracts of the 4 mosquito species may be seen in Table 2, as well as in Table 3, which presents the protein profiles for WBE for the 4 mosquito species.

A comparative analysis of the polypeptides revealed markedly different patterns in mosquito saliva, salivary gland extracts and whole body extracts. Although the differences in frequencies were not significant, SGE had slightly more polypeptide bands than either of the other two extracts.

Identification of major allergens in 4 mosquito species

The major allergens in the extracts which elicit specific IgE responses in the pooled sera of subjects allergic to mosquito bites are shown in Figure 2 and Table 4.

The current study documents 16, 6 and 2 major allergens detected from saliva, SGE and WBE respectively, for the 4 mosquito species. Saliva contained a greater number of allergens compared with the other extracts.

The species-shared and species specific allergens differentiated by MW (kDa) detected in saliva, SGE and whole body extracts for the 4 mosquito species are presented in Table 4. In the saliva of the 4 mosquito species, an allergen with a molecular weight of 75.4 kDa was found in *Cx. quinquefasciatus* and *Ae. Aegypti*, while 49.5 and 36 kDa allergens were found in *Cx. quinquefasciatus Ae. aegypti*. and *An. minimus.* A 32-kDa allergen was found in *Cx. quinquefasciatus* and *An. minimus*. A 22 kDa allergen was found in *Cx. quinquefasciatus*, *Ae. albopictus*, *An. minimus*. Allergens with a MW of 37.5 kDa were found in SGE of all mosquito species while the 35.5 kDa allergen was found in both *Ae. Aegypti* and *An. minimus.* No shared allergen was found in the WBE of the 4 mosquito species.

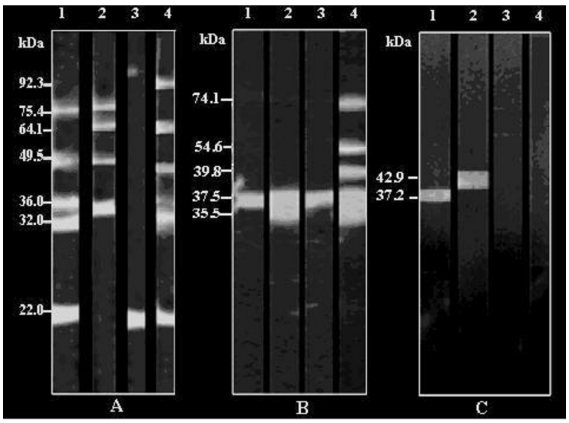

Figure 2. Allergen profile patterns of saliva (panel A), salivary gland extracts (panel B) and the whole body extracts (panel C) where lane 1 = *Cx. quinquefasciatus*, lane 2 = *Ae. aegypti,* lane 3 = *Ae. albopictuus* and lane 4 = *An. minimus*

A species-specific allergen with a MW of 92.3 kDa was detected in saliva and those allergens with MW of 42.9 and 37.2 kDas were found in the WBE.

The mean frequency of each of the analyzed extracts over the 4 species of mosquitoes

Figure 3 presents the mean number of proteins and allergens detected with the combined totals of the 4 mosquito species. On the average, many more proteins were observed in the SDS-PAGE than allergens for the 3 extracts. There were no significant differences in the proteins (P = 0.68) even though SGE had a higher number of proteins than either of the other two extracts. For the detected allergens, the number found in saliva was greater than the frequencies in WBE and SGE, although the differences were not statistically different (P = 0.85).

Discussion

For the first time in Thailand and, by extension, South East Asia, proteins and allergens prominent in the sera of subjects with mosquito bite allergies have been described for four mosquito species; *Culex quinquefasciatus, Aedes aegypti, Aedes albopictus and Anopheles minimus*. A comparative analysis of the polypeptides revealed markedly different patterns in mosquito saliva, salivary gland extracts and whole body extracts. SGE had slightly more polypeptide bands than saliva. These results confirm previous finding that SGE contains a number of proteins which are not secreted in the

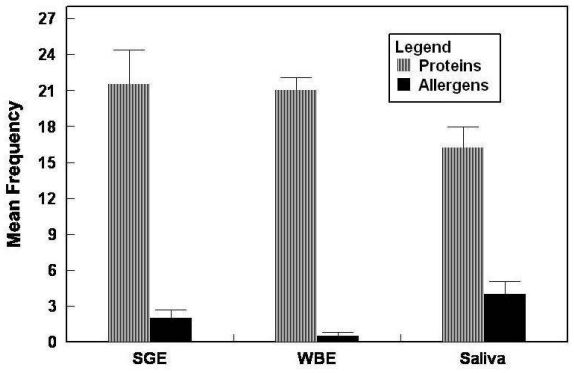

Figure 3. The mean frequency of each of the analyzed extracts for the 4 species of mosquitoes

saliva[24, 25] The results of the present study confirm the previous findings that commercially available mosquito whole-body extracts contain many extraneous proteins that are not present in mosquito saliva, and might interfere with diagnostic skin testing or *in vitro* tests in subjects with a history of allergic reactions to mosquito bites.[14] Immunotherapy using injections of mosquito whole body extract has been reported to prevent allergic reaction to subsequent bites. [26, 27] Nevertheless, the therapy is not widely used in the treatment of mosquito allergies because commercially available mosquito whole body extract are ineffective in down regulating the specific immune responses to mosquito salivary allergens and may even cause additional sensitization.[14]

The results from the present study suggest that saliva is the best allergen sources for diagnostic and for immunotherapy of mosquito allergies. However, saliva production from living mosquitoes is tedious and time consuming. An alternative is molecular cloning of mosquito saliva allergens. This is a powerful tool for large scale production of pure and safe mosquito salivary allergens which is beneficial for standardization, effective diagnosis and improved specific immunotherapy for patients with systemic reactions to mosquito bites.[9] The identification of major allergens facilitates the production of specific recombinant allergens. Recently, a recombinant *Aedes aegypti* salivary allergen, rAed a 1, rAed a 2 and rAed a 3 has been expressed, purified, characterized and used in *in-vitro* diagnosis of mosquito allergies. Immunoassays using recombinant mosquito salivary allergens,

Table 4. MW (kDa) of the major allergens in the saliva, SGE and WBE delineated by mosquito species which elicit specific IgE responses in pooled sera of subjects allergic to mosquito bites.

Type of Extract	Major allergen	Mosquito Species			
Saliva	92.3 kDa	-	-	-	*An. minimus*
	75.4 kDa	*Cx. quinquefasciatus*	*Ae. aegypti*	-	-
	64.1 kDa	-	*Ae. aegypti*	-	*An. minimus*
	49.5 kDa	*Cx. quinquefasciatus*	*Ae. aegypti*	-	*An. minimus*
	36.0 kDa	*Cx. quinquefasciatus*	*Ae. aegypti*	-	*An. minimus*
	32.0 kDa	*Cx. quinquefaciatus*	-	-	*An. minimus*
	22.0 kDa	*Cx. quinquefascitus*	-	*Ae. albopictus*	*An. minimus*
SGE	37.5 kDa	*Cx. quinquefasciatus*	*Ae. aegypti*	*Ae.albopictus*	*An. minimus*
	35.5 kDa	-	*Ae. aegypti*	-	*An. minimus*
WBE	42.9 kDa		*Ae. aegypti*	-	-
	37.2 kDa	*Cx. quinquefasciatus*	-	-	-

especially rAed a 1 and rAed a 3, are sensitive and specific for the diagnosis of mosquito allergy.[3,28]

In the pooled sera of the study subjects, the specific IgE antibodies were not only bound to salivary allergens of the 3 human biting species, but also bound to the allergens of *An. minimus*, which is a zoophilic strain. These findings suggest that sensitization of allergic subjects by mosquito bites from one species can confer reactivity against another species. These species-shared allergens hold great promise for the diagnosis and specific immunotherapy of patients' subject to exposure to a wide range of mosquito species. Using allergens from one species may provide protection/immunology against reactions triggered by another species.[15] Moreover, using combination of major allergens will provide a greater opportunity for a therapeutic cocktail to be successful in ameliorating cutaneous reactions in patients reactive to mosquito bites.

The present study revealed the major allergens from various extracts of the 4 mosquito species found in Thailand which induce specific IgE response in the sera of Thai subjects with mosquito allergy. Both species-specific and species-shared allergens of the 4 mosquito species were identified. Species-shared allergens with MW of 75.4, 49.5, 36, 32 and 22 kDas have been identified as major allergens in the saliva of *Cx. quinquefasciatus*. Since this species is the most common in Thailand, further production of recombinant allergens on the basis of the major allergens identified in the present study, i.e. saliva allergens with MW of 36, 32 and 22 kDa, should be pursued. The shared allergens with MW of 75.4 and 49.5 kDas might not be useful because in our previous study, specific IgE antibodies against several saliva proteins of *Cx. quinquefasciatus* with high molecular weight ranging from 45 kDa were seen in both mosquito bite allergic patients and non-allergic subjects.[10]

The major allergens of the mosquitoes' saliva observed in mosquito allergic subjects from Manitoba[15] were different from the major allergens observed in the present study. This may indicate that the saliva of the same mosquito species may elicit different immune responses in populations differing in age, gender, ethnicity and/or genomic structure. These results clearly suggest a commonality of clinical responsiveness

to mosquito bites engendered by cross-reactivity due to similar epitopes in the saliva of phylogenetically related mosquito species. Thus, homologous allergens may not only confer structural cross-reactivity, but may also bestow functional responsiveness, which is not an uncommon theme in allergy.[29] Discriminating proteomic tools could achieve the functional characterization of the detected allergens.

A previous study reported that some allergens in *Ae. togoi* were not identified in the pooled serum from subjects allergic to mosquitoes obtained from North America because *Ae. togoi* is distributed only in eastern Asia.[3] Thus, the identity of major allergens from mosquito species in different geographic regions is relevant and must be considered before making unwarranted generalizations.

It may be concluded that: (a) the protein profiles of saliva, SGE and WBE of the mosquito species found in Thailand and/or SEA were successfully characterized with SGE yielding the greater number followed by WBE and saliva; (b) the major allergens in the extracts which elicit specific IgE responses in pooled sera of subjects allergic to mosquito bite were successfully identified with saliva having the most allergens followed by SGE and WBE; (c) for immunotherapeutic purposes, it is suggested that saliva should be the vehicle of choice; (d) WBE should not be used as a source of allergens since it has few allergens.

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