

Comparison of proteins, IgE, and IgG binding antigens, and skin reactivity in commercial and laboratory-made mosquito extracts

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Background: Commercial extracts are available for the diagnosis and treatment of mosquito allergy, but their antigen content has never been analyzed.

Objective: We wanted to analyze commercially available mosquito extracts and to compare these extracts with different laboratory preparations.

Methods: Seven commercially available mosquito whole body extracts from six companies and four laboratory mosquito preparations including saliva extract were studied. Epicutaneous tests and measurement of protein concentration were performed. Protein components were identified by sodium sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver stain. IgE and IgG binding antigens were analyzed by SDS-PAGE and immunoblotting with sera from mosquito-allergic subjects.

Results: The seven commercial materials produced wheals and papules ranging from 0 to 36 mm². Their protein concentrations varied from 0.1 to 4.9 mg/mL. There were significant differences in their protein and antigen components. Some extracts contained multiple highly immunoreactive proteins and IgE- and IgG-binding antigens that are not present in mosquito saliva, but few actual salivary antigens. In the four laboratory preparations, rank ordered from whole body, head and thorax, salivary gland to saliva extracts, the amount of salivary antigens significantly increased, while non-salivary proteins and antigens significantly decreased.

Conclusions: Commercial mosquito extracts should be standardized. Purer mosquito extracts should be used in diagnosis and immunotherapy of mosquito allergy.

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INTRODUCTION

Allergic reactions to mosquito bites are a common problem world-wide. Although cutaneous reactions are seen most often, systemic reactions including generalized urticaria and angioedema, rhinitis, conjunctivitis, and/or asthma have been reported.¹⁻³ Anaphylactic shock following mosquito bites has been also reported.⁴

Immunotherapy is successful in the treatment of mosquito allergy,^{1,2,4-6} correlating with the observation that

natural desensitization eventually occurs during long-term exposure to the bites.⁷⁻⁹ This therapy is neither well studied nor widely used at least in part because commercially available mosquito extracts have never been characterized and standardized. In order to improve the precision of diagnosis of mosquito allergy, and to ensure the efficacy and safety of immunotherapy with mosquito extracts, commercial mosquito extracts should be analyzed with respect to protein and antigenic composition, and biologic activity. In this study we investigated seven commercially available mosquito whole body extracts supplied for skin testing and/or immunotherapy from six manufacturers. We compared the commercial materials with four mosquito extracts made in our laboratory from mosquito whole body, head and tho-

rax, salivary gland, and saliva. We performed epicutaneous tests, measurement of protein concentration, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein components, and SDS-PAGE and immunoblotting for IgE- and IgG-binding antigens with all commercial and laboratory-made extracts.

MATERIALS AND METHODS

Commercial Mosquito Extracts

Seven commercial mosquito extracts available for epicutaneous tests and/or immunotherapy in mosquito allergy were purchased from six manufacturers. These extracts were made from *Culex* and *Aedes* genera. Information provided by the manufacturers about the extracts is listed in Table 1.

Laboratory-Made Mosquito Aedes aegypti Preparations

An *Aedes aegypti* mosquito colony was obtained from the Department of Entomology, University of Manitoba, and maintained in our laboratory. Female mosquitoes were used in the preparation of mosquito whole body, head and thorax,¹⁰ salivary gland,¹¹ and saliva extracts. Saliva was collected from 3 to 15-day-old adult mosquitoes by placing the proboscis of each mosquito into a capillary tube filled with water. Salivation was induced by applying 0.5 µL of 0.5% malathion in acetone to the thorax.¹² One hour later, the contents of capillary tubes containing saliva were collected, pooled, and lyophilized. The saliva was reconstituted by dissolving the lyophilized proteins in 0.02 M phosphate buffered saline.

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Table 1. Commercial Mosquito Extracts and Information Provided by Manufacturers

No	Name of Manufacturer	Lot No	Species	Concentration	Preservative	Use
C1	Hollister-Stier (Miles Canada Inc, Etobicoke, Ont)	D41J3173	<i>Aedes aegypti</i>	1:10 wt/vol	50% glycerin	Epicutaneous test
C2	Hollister-Stier (Miles Canada Inc, Etobicoke, Ont)	H42J5370	<i>Culex pipiens</i>	1:10 wt/vol	50% glycerin	Epicutaneous test
C3	ALK Labs, Inc (Berkeley Biologicals, Berkeley, CA)	635090	<i>Aedes</i> species	1:50 wt/vol	50% glycerin 0.5% phenol	Epicutaneous test
C4	Allergy Labs of Ohio Inc (ALO) (Columbus, OH)	B406212A	<i>Culex pipiens</i>	1:20 wt/vol	0.4% phenol	Epicutaneous test Immunotherapy
C5	Centre Labs (Port Washington, NY)	4G66391	<i>Culex pipiens</i>	1:10 wt/vol 20K PNU/mL	0.4% phenol	Immunotherapy Epicutaneous test
C6	Meridian (Subsidiary of Ventrex Labs, Inc) (Round Rock, TX)	3V1	<i>Culex pipiens</i>	1:10 wt/vol	50% glycerin	Epicutaneous test
C7	Greer Labs, Inc (Lenoir, NC)	B10-33	<i>Aedes taeniorhynchus</i>	1:10 wt/vol 20K PNU/mL	0.4% phenol	Epicutaneous test

Epicutaneous Tests and Mosquito Bite Tests

Epicutaneous tests with the seven commercial mosquito extracts and the four laboratory mosquito preparations were performed on two subjects with severe skin reactions to *Aedes aegypti* bites and on two subjects with no skin reaction to the bites. These subjects were selected from 41 subjects studied.⁹ Histamine phosphate (1 mg/mL), saline and 50% glycerin in saline were used as positive and negative controls, respectively. Mosquito bite tests were performed using female mosquitoes (*Aedes aegypti*) reared in our laboratory. Immediate wheal and flare reactions were measured 30 minutes after the tests and delayed papule reactions were measured 24 hours later using the largest and the orthogonal diameters of the wheal or the papule, respectively. The area of the wheal or papule was calculated after subtracting the area of the wheal or papule produced by the relevant negative control (if present).

Protein Assay

Protein concentration of each commercial mosquito extract and laboratory mosquito preparation was determined by a Bio-Rad Protein Assay kit (Bio-Rad Labs, Richmond, CA).

SDS-PAGE and Silver Stain

Proteins from mosquito extracts were separated by SDS-PAGE under reducing conditions in a discontinuous system using a Bio-Rad mini slab gel ap-

paratus as described previously.¹⁰ One to two micrograms each of the laboratory-made mosquito whole body, head and thorax, salivary gland, and saliva extracts, were loaded onto different wells and electrophoresed in 10% acrylamide SDS-PAGE. Molecular weight protein standards (Bio-Rad) were used to determine the relative molecular weights of the electrophoresed components. Separated proteins were detected by silver staining (Bio-Rad Silver Stain kit).

SDS-PAGE and Immunoblot Analysis of IgE and IgG Binding Antigens

The pooled serum used in immunoblotting was obtained from six subjects exhibiting severe skin reactions to mosquito bites and high mosquito-specific IgE and IgG as measured by ELISA.¹¹ These subjects, including the two subjects used in the skin and bite tests, lived in Manitoba or Texas where the *Aedes* and/or *Culex* mosquito species are abundant.

SDS-PAGE and immunoblot were performed as described previously.¹⁰ In the comparison of commercial mosquito extracts, 10 μ L of each commercial extract, 6 μ L of *Aedes aegypti* saliva and 10 μ L of *Culex quinquefasciatus* salivary gland extract were loaded onto different wells. In the comparison of different mosquito preparations, proteins were loaded as indicated in Figure 3. Proteins separated by 12% SDS-PAGE were elec-

trophoretically transferred onto nitrocellulose membranes. Free binding sites on the membranes were blocked by incubation with 3% bovine serum albumin in 0.05 M PBS Tween 20 for two hours. Immunoblotting was completed by incubation of the membranes with the pooled serum (1:10 dilution for IgE binding antigens, 1:50 for IgG binding antigens). After washing, this was followed by sequential incubations of the membranes with monoclonal anti-human IgE (clone #7.12 from Dr. A. Saxon's laboratory) or monoclonal anti-human IgG (PharMingen, CA), and HRP-conjugated goat anti-mouse IgG (Calbiochem Corporation, CA). After washing, the membranes were finally incubated with ECL detecting reagents (Amersham Life Science, Buckinghamshire, England) and then exposed to the film (X-Omat, Kodak). Pre-stained SDS-PAGE standards (Bio-Rad, Richmond, CA) were used to determine the relative molecular weights of the electrophoresed components.

RESULTS

Epicutaneous Tests, Mosquito Bites Tests, and Protein Concentrations

Immediate and delayed reactions were found at the sites of skin tests and mosquito bites in the two subjects allergic to mosquito bites. The immediate reaction was a pruritic wheal with a surrounding flare appearing within a few minutes, peaking at 30 minutes

and then subsiding. The delayed papules were found several hours later, reaching a peak 24 hours after the epicutaneous and the bite tests. In contrast, there was little or no skin reaction to either the epicutaneous sites or the bite site in the two control subjects.

Wide variations in both skin immediate and delayed reactions to the seven commercial mosquito materials were found in the two reactive subjects. The mean immediate wheal sizes ranged from 0.5 to 32 mm², and the mean delayed papule sizes ranged from 0 to 36 mm² (Table 2). Two aqueous extracts, C5 and C7, appeared to have no biologic activity in humans. The protein concentration of the commercial extracts varied greatly, ranging between 0.09 and 4.85 mg/mL, but generally correlated with the size of skin reactions ($r = .72$, $P < .003$ for wheal; $r = .80$, $P < .005$ for papule). An exception to this was the C7 extract which had a protein concentration of 1.67 mg/mL, but elicited no skin response.

In order to evaluate the biologic activity of each gram of protein, the ratio of skin reactions/protein concentration was calculated (Table 2). The skin reactivity per gram of protein varied from 0.3 to 14.9 in the seven commercial extracts. Among the four laboratory-made mosquito preparations, as the

purity of the preparation increased, rank ordered from whole body, head and thorax, salivary gland to saliva, the skin reactivity per gram of protein increased significantly. Perhaps the skin reactivity per gram of protein in saliva (78.0) was less than that found in salivary gland (128.6) because the lyophilization required in the preparation of saliva extract reduced some biologic activity of saliva.

SDS-PAGE and Silver Stain

In the four laboratory preparations, rank ordered from whole body, head and thorax, salivary gland to saliva extracts, the amount of salivary antigens significantly increased, while nonsalivary proteins and antigens significantly decreased (Fig 1). There were 24 visible protein bands in the head and thorax extract, 16 in the salivary gland extract, but only 9 in the saliva extract.

When commercial extracts containing the same amount of protein were used, the protein bands were obscure and the background was very dark, therefore, these results are not included.

SDS-PAGE and Immunoblot Analysis

The IgE and IgG binding antigens in the seven commercial mosquito extracts are shown in Figure 2. The anti-

gens of the three commercial *Aedes* extracts are compared with the antigens in the *Aedes aegypti* saliva extract, while the antigens of the four commercial *Culex* extracts are compared with the antigens in the *Culex quinquefasciatus* salivary gland extract. *Culex quinquefasciatus* and *Culex pipiens* belong to the same genus and are sibling species. Because the *Culex* species does not salivate using the method applied to induce *Aedes* species to salivate, the salivary gland extract of *Culex quinquefasciatus* was used to compare antigens with the *Culex pipiens* whole body extracts. Among the seven commercial mosquito extracts both IgE and IgG binding antigens varied greatly both in the number of antigen bands and the amount of each antigen. Multiple antigens were found in the commercial extracts. Most of these antigens were not present in the saliva or salivary gland extract. Some extracts (C1, C2, and C3) contained small amounts of saliva antigens. Two extracts, C5 and C7, contained no visible saliva or salivary gland antigens although they did contain other proteins (0.09 and 1.67 mg/mL of proteins, respectively).

The IgE and IgG binding antigens in one commercial mosquito whole body extract and four laboratory-made mosquito preparations (whole body, head

Table 2. Protein Concentrations and Epicutaneous Test Results of Commercial and Laboratory Mosquito Extracts

Mosquito Extracts, wt/vol	Protein Concentration, mg/mL	Skin reactions (mm ²)		Skin reaction/Protein	
		Immediate Wheal	Delayed Papule	Immediate Wheal	Delayed Papule
Commercial whole body extracts					
C1 <i>Aedes aegypti</i> (1:10)	3.16	7.5 (2)*	3.0 (0)	2.4	0.9
C2 <i>Culex pipiens</i> (1:10)	2.58	14.5 (0.5)	14.0 (0)	5.6	5.4
C3 <i>Aedes</i> species (1:50)	1.16	10.5 (0)	5.0 (0)	9.1	4.3
C4 <i>Culex pipiens</i> (1:20)	1.24	18.5 (0)	10.0 (0)	14.9	8.1
C5 <i>Culex pipiens</i> (1:10)	0.09	0.5 (0)	0.0 (0)	5.6	–
C6 <i>Culex pipiens</i> (1:10)	4.85	32.0 (5)	36.0 (0)	6.6	7.4
C7 <i>Aedes taeniorhyncus</i> (1:10)	1.67	0.5 (0)	0.0 (0)	0.3	–
Laboratory-made extracts of <i>Aedes aegypti</i>					
Whole body	1.20	11.0 (0)	3.0 (0)	9.2	2.5
Head and thorax	0.62	11.0 (0)	1.0 (0)	17.7	1.6
Salivary gland	0.14	18.0 (0.5)	2.0 (0)	128.6	14.3
Saliva	0.25	19.5 (0)	6.5 (0)	78.0	26.0
<i>Aedes aegypti</i> bite		80 (0)	367 (0)	–	–

* Mean reaction size in the controls.

and thorax, salivary gland, and saliva) are shown in Figure 3. Whole body and head and thorax extracts contained multiple antigens which were not present in saliva, but they contained few of the antigens present in saliva, although more micrograms of proteins were loaded for these extracts. As the purity of the preparation increased from whole body extract to saliva, the number of proteins (Fig 1) and antigens (Fig 3) decreased dramatically. This correlates with the results shown in Table 2, in which the salivary gland and saliva extracts have lower protein concentrations (0.14 and 0.25 mg/mL, respectively) and higher ratios of skin reactivity/protein (128.6 and 78.0, respectively) than those in the whole body (protein 1.2 mg/mL, ratio 9.2) and the head and thorax extracts (protein 0.62 mg/mL, ratio 17.7).

Inter-Lot Variation

In order to examine inter-lot variation, we examined the two extracts from different lots of the *Culex pipiens* made by Hollister-Stier (lot No. H42J5370 and F46E2416) for protein concentration, skin reactivity (on two subjects), and IgE and IgG binding property. The results of the two extracts were very similar, 2.6 and 2.7 mg/mL for protein concentration, 11.5 and 14 mm² for wheal size and 40 and 49 mm² for delayed papule, respectively, and the same spectrum in the immunoblot, suggesting that the inter-lot variation of the Hollister-Stier extracts is small.

DISCUSSION

The commercially available mosquito extracts used in the diagnosis and treatment of mosquito allergy have not been analyzed previously. As mosquito saliva has been demonstrated to be the source of the antigens which produce typical bite reactions in man,¹³ we used mosquito saliva or salivary gland extracts as standard mosquito antigen references in our analysis of seven commercially available mosquito extracts. Because mosquito-specific IgE and IgG antibodies were found to be involved in the develop-

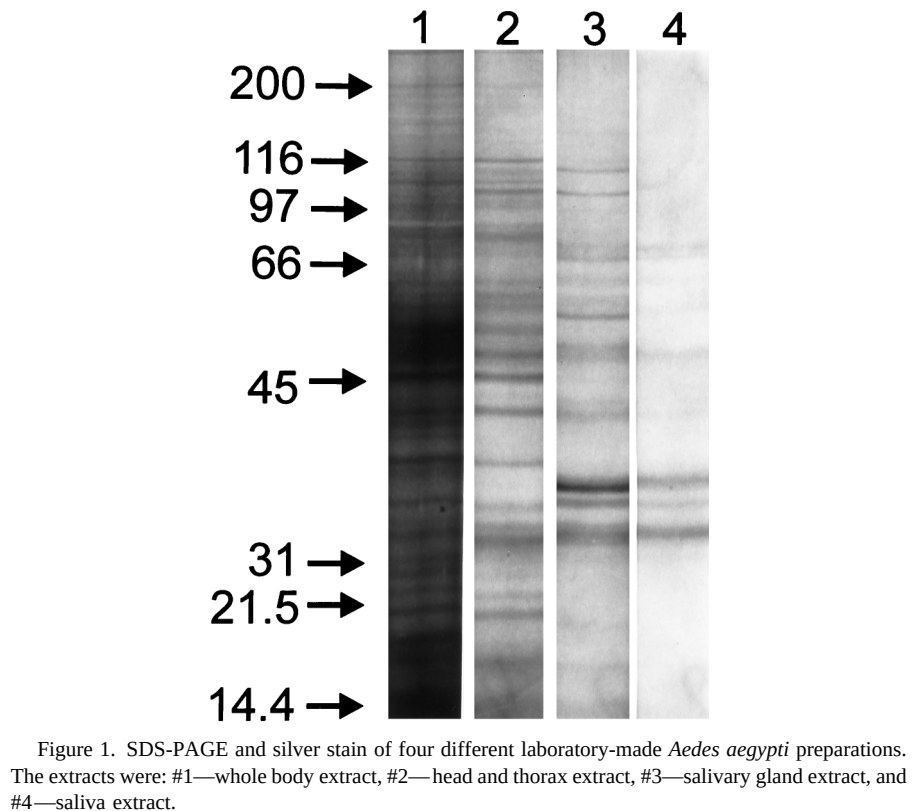


Figure 1. SDS-PAGE and silver stain of four different laboratory-made *Aedes aegypti* preparations. The extracts were: #1—whole body extract, #2—head and thorax extract, #3—salivary gland extract, and #4—saliva extract.

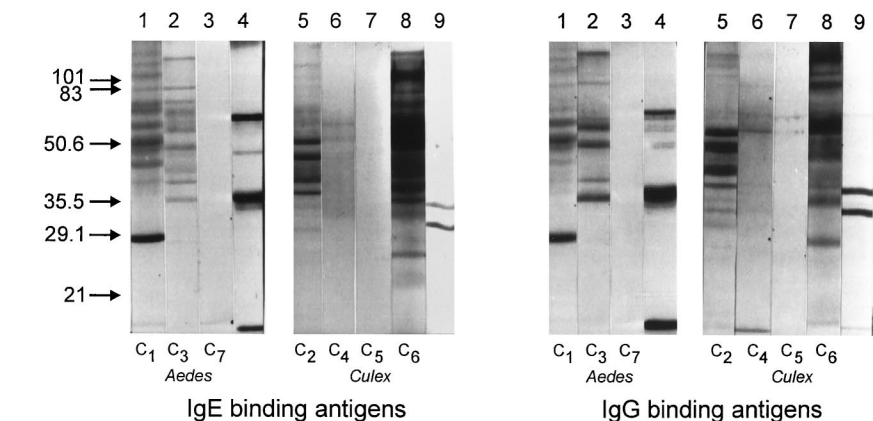


Figure 2. Immunoblot analysis of IgE and IgG binding antigens in seven commercial mosquito whole body extracts. Ten microliters of each mosquito extract was analyzed by SDS-PAGE and immunoblotting as described in the "Methods." The extracts were: #1: C1—Hollister-Stier; #2: C3—ALK; #3: C7—Greer; #4: *Aedes aegypti* saliva collected in our lab; #5: C2—Hollister-Stier; #6: C4—ALO Labs; #7: C5—Centre Labs; #8: C6—Meridian; #9: *Culex quinquefasciatus* salivary gland extract prepared in our lab. To the left are IgE binding antigens and to the right are IgG binding antigens. Molecular weight standards are indicated.

ment of mosquito allergy,^{9–11,14–17} we analyzed IgE and IgG binding antigens in these mosquito extracts.

Using both in vitro and in vivo tests, we found considerable variation in the

commercial mosquito extracts. Indeed, two (C5 and C7) of three aqueous extracts (C4, C5, C7) elicited neither immediate nor delayed skin reactions and presented no visible antigen band in

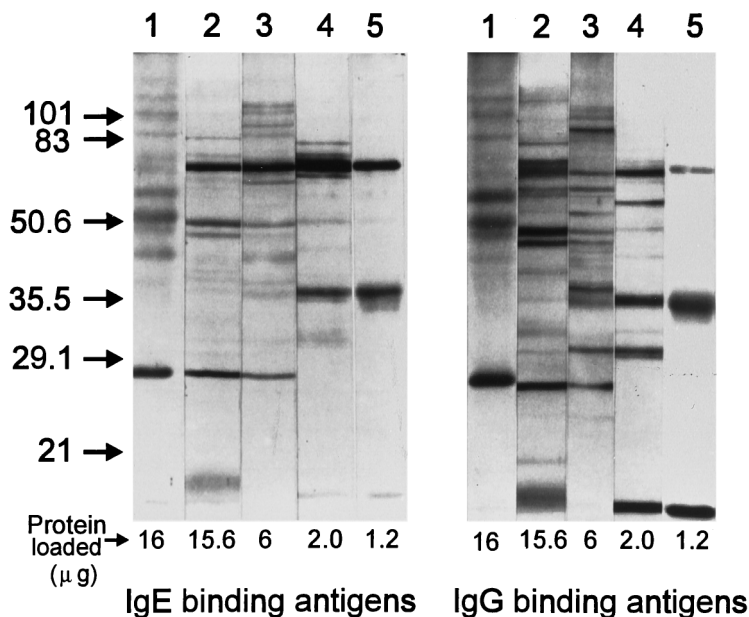


Figure 3. Immunoblot analysis of IgE and IgG binding antigens in different preparations of *Aedes aegypti*. The extracts were: #1: C1—*Aedes aegypti* whole body extract from Hollister-Stier; #2–#5: different *Aedes aegypti* preparations made in our lab; #2: whole body extract; #3: head and thorax extract; #4: salivary gland extract; #5: saliva. To the left are IgE binding antigens and to the right are IgG binding antigens. Molecular weight standards and protein amounts loaded are indicated.

immunoblot (Fig 2). All four extracts preserved by 50% glycerin exhibited skin reactivity and visible protein bands suggesting that the extracts preserved in 50% glycerin are more potent and stable.

We also found that the commercial mosquito extracts contained multiple proteins and antigens, many of which are unrelated to the antigens in mosquito saliva. The antibodies directed against these non-saliva antigens in the commercial extracts may have been induced by inhalation of insect particles or by being bitten by other insects whose antigens cross-reacted with mosquito body components leading to the formation of IgE and IgG antibodies against mosquito body antigens. Cross-reactivity is widespread in nature and has even been reported between chironomids and shrimp.¹⁸ A 37-kDa IgE binding antigen has been found to be shared by six Diptera species.¹⁹

Our results suggest that commercial mosquito extracts should be standard-

ized and that purer mosquito extracts should be used in the diagnosis and immunotherapy of mosquito allergy. In addition to having poor diagnostic accuracy in skin testing, injection of crude extract may lead to the development of sensitization to some of its components.²⁰ It has been demonstrated that the efficacy of immunotherapy is considerably improved by the use of purified antigens.²¹ For example, immunotherapy with purified Hymenoptera venom protects 97% of subjects with systemic reactions to Hymenoptera from future anaphylaxis from stings.²²

Because the process of collecting or extracting mosquito saliva is extremely tedious, it is currently impractical to use mosquito saliva or salivary gland extracts in diagnosis or immunotherapy. It is therefore both feasible and important to standardize commercially available mosquito extracts to increase the quantity of the active materials they contain. Standardization of antigen preparation is usually achieved by

comparison of overall activity or major components of the extract with those of a reference preparation. International standards are now available for a number of antigens such as weed pollen, tree pollen, grass pollen, house dust mites, animal danders, and molds.²³ An in-house reference mosquito extract and a pooled allergic serum for each mosquito genus should be used to evaluate the relative biologic activity and the lot-to-lot variation of different batches of mosquito extracts. Antigen extracts vary not only in total potency but also in the distribution of the identifiable antigen components as demonstrated in the present report (Figs 2 and 3) and other studies.²⁴ The major antigen content of allergic preparations has been demonstrated to reflect biologic activity and has been recommended to standardize antigen extracts.²⁵ In mosquito allergy, species-shared antigens have been reported.^{10,17,26,27} Our recent study showed that a recombinant 37-kDa protein in *Aedes aegypti* salivary glands²⁸ was shared by all five *Aedes* species studied and by *Culex quinquefasciatus* as well.²⁷ This provides evidence that standardization of mosquito extracts can be achieved by the use of a few major species-shared antigens.

Characterization and recombination of the major mosquito saliva antigens and production of monoclonal antibodies to the antigens are currently ongoing in our laboratory. In the future, these major antigens and their specific monoclonal antibodies will provide more accurate and useful information for analysis and standardization of mosquito extracts, improving diagnosis and immunotherapy in mosquito allergy.

In the present study, we have shown that mosquito head and thorax extract contained more saliva proteins and antigens than whole body extracts (Figs 1 and 3) and was easier to prepare than salivary gland or saliva extracts. In the interim, we suggest the use of this extract instead of whole body extract for diagnosis and immunotherapy of mosquito allergy.

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