



Allergens involved in the cross-reactivity of *Aedes aegypti* with other arthropods

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ABSTRACT

Background: Cross-reactivity between *Aedes aegypti* and mites, cockroaches, and shrimp has been previously suggested, but the involved molecular components have not been fully described.

Objective: To evaluate the cross-reactivity between *A aegypti* and other arthropods.

Methods: Thirty-four serum samples from patients with asthma and/or allergic rhinitis were selected, and specific IgE to *A aegypti*, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blomia tropicalis*, *Periplaneta americana*, and *Litopenaeus vannamei* was measured by enzyme-linked immunosorbent assay. Cross-reactivity was investigated using pooled serum samples from allergic patients, allergenic extracts, and the recombinant tropomyosins (Aed a 10.0201, Der p 10, Blo t 10, Lit v 1, and Per a 7). Four IgE reactive bands were further characterized by matrix-assisted laser desorption/ionization tandem time of flight.

Results: Frequency of positive IgE reactivity was 82.35% to at least one mite species, 64.7% to *A aegypti*, 29.4% to *P americana*, and 23.5% to *L vannamei*. The highest IgE cross-reactivity was seen between *A aegypti* and *D pteronyssinus* (96.6%) followed by *L vannamei* (95.4%), *B tropicalis* (84.4%), and *P americana* (75.4%). Recombinant tropomyosins from mites, cockroach, or shrimp inhibited the IgE reactivity to the mosquito at a lower extent than the extracts from these arthropods. Several bands of *A aegypti* cross-reacted with arthropod extracts, and 4 of them were identified as odorant binding protein, mitochondrial cytochrome C, peptidyl-prolyl cis-trans isomerase, and protein with hypothetical magnesium ion binding function.

Conclusion: We identified 4 novel cross-reactive allergens in *A aegypti* allergenic extract. These molecules could influence the manifestation of allergy to environmental allergens in the tropics.

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Introduction

Mosquitoes are distributed worldwide and may induce allergic reactions in atopic individuals.¹ Sensitization to mosquito bites is common in the United States, Canada, Japan, Switzerland, and Finland,^{2–4} sometimes causing severe systemic reactions, such as generalized urticaria, angioedema, wheezing, and even anaphylaxis.^{1,5,6} Several allergens from the genera *Aedes*, *Culex*, and

Anopheles have been identified, and 4 saliva-derived allergens from the species *Aedes aegypti* have been reported.^{7–10}

In the tropics and subtropics, sensitizations to mites, cockroaches, and shrimp are highly prevalent, being important inducers of allergic reactions.^{11–18} *A (Stegomyia) aegypti*, originally from Africa, has evolved to coexist with humans and thrives in a wide range of climates and habitats,^{19–21} including tropical and subtropical regions.^{19,22–24} Mosquito detritus present in house dust could also be inhaled,^{25,26} leading to sensitization and induction of allergic respiratory symptoms in sensitized patients.

Tropomyosin is a panallergen that belongs to a family of phylogenetically conserved proteins with multiple isoforms present in muscle and nonmuscle cells of vertebrates and invertebrates.^{27–31} We have identified that *A aegypti* expresses several tropomyosin variants that cross-react with tropomyosin from other arthropods.³² However, other allergens involved in this cross-reactivity with other arthropods remain to be discovered.

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Several allergens have been described in arthropods, including tropomyosins, myosins, paramyosins, fatty acid-binding proteins, glutathione S-transferase, arginine kinase, sarcoplasmic calcium-binding protein, and hyaluronidase, among others (www.allergome.org and www.allergen.org). *A. aegypti* has genes that encode homologues of the previously mentioned allergens. Our main hypotheses were that a high cross-reactivity between *A. aegypti* and arthropods may exist and that several molecules, in addition to tropomyosin, are involved. The aim of this study was to evaluate the components involved in a potential cross-reactivity between *A. aegypti* and other arthropods.

Methods

Serum Samples

Serum samples were obtained from 34 patients (15 males and 19 females; mean [SD] age, 15.4 [13.2] years) who attended an outpatient allergy clinic in Fort de France, Martinique, France (Table 1). All patients were skin prick tested with a battery of common standardized inhalant allergens (Stallergènes, Antony, France). Individuals who developed a wheal larger than 3 mm to the extracts used were considered as having a positive skin prick test (SPT) result. Serum was collected after written consent was obtained from the participants in the study entitled: “Recherche des causes allergiques de l’asthme en Martinique.” This study was approved by the “Caisse generale de securite sociale de la

Martinique: DAAM-JP/RJ” and sponsored by the Fond’s d’Aide a la Qualite des Soins de Ville, Fort de France, Martinique.

Preparation of Allergenic Extracts

All the allergenic extracts, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blomia tropicalis*, *Periplaneta americana*, *Betula verrucosa*, *Litopenaeus vannamei*, and *A. aegypti*, were prepared at Immunotek S.L. Laboratories, Madrid, Spain. All source materials were extracted overnight in 0.1 M phosphate-buffered saline (PBS) (at 1:20 w/v) at 4°C in constant stirring. After extraction, the mixture was centrifuged at 7,840g for 10 minutes and the supernatant filtered through a 0.2-μm membrane. After filtration, the extracts were dialyzed against distilled water in 3,500-Da cut-off membrane (Spectra/Por Dialysis membranes, Houston, Texas) and lyophilized. Protein content was measured by the Bradford method.

Recombinant Arthropod Tropomyosins

His-tagged recombinant tropomyosins from *A. aegypti* (Aed a 10.0201) and *L. vannamei* (Lit v 1) were obtained by expression in *Escherichia coli* and purified by affinity chromatography using Ni-NTA resin.³² Production of recombinant tropomyosin from *D. pteronyssinus* (Der p 10) and *B. tropicalis* (Blo t 10) was performed as described previously.³³ Recombinant tropomyosin from *P. americana* (Per a 7) was purchased from Indoor Biotechnologies (Cardiff, UK).

Table 1
IgE Levels to Allergenic Extracts in the Serum Samples From the Studied Population^a

Patient no.	Age, y	Sex	<i>Aedes aegypti</i>	<i>Dermatophagoides pteronyssinus</i>	<i>Dermatophagoides farinae</i>	<i>Blomia tropicalis</i>	<i>Periplaneta americana</i>	<i>Litopenaeus vannamei</i>	Total IgE, IU/mL
1 ^b	5.62	M	0.319	0.047	0.052	0.045	0.078	0.050	324
2	32.1	F	2.810	1.404	0.937	0.207	0.155	0.843	466
3 ^b	16.8	M	0.795	0.070	0.065	0.052	0.043	0.045	1199
4	39.08	F	0.065	0.187	0.358	0.398	0.053	0.048	243.86
5	8.4	M	0.142	0.210	0.209	0.749	0.049	0.059	752
6	21	F	0.079	0.204	0.469	0.328	0.062	0.044	306
7	11	M	0.070	0.931	1.825	0.666	0.047	0.044	2030
8	4.17	F	0.097	0.678	0.805	0.338	0.054	0.047	835
9	4.07	M	0.058	0.522	0.295	0.130	0.046	0.042	1067
10	5.61	F	0.248	0.231	2.661	2.330	0.047	0.044	1474
11	6.18	M	0.178	0.062	0.109	0.257	0.056	0.053	697
12 ^b	12.48	F	0.232	0.042	0.046	0.043	0.043	0.048	879
13	4.13	M	0.162	0.303	0.523	0.161	0.462	0.123	1496
14	15.09	M	1.240	1.373	1.820	0.272	0.092	0.509	1246
15 ^c	47.9	F	0.179	0.070	0.080	0.046	0.259	0.053	107
16	25.38	F	0.063	0.771	3.079	3.947	0.050	0.044	1198
17	35.66	F	0.055	0.071	0.068	0.124	0.045	0.053	267.86
18	9.73	M	0.060	0.674	1.142	0.048	0.048	0.051	210
19	39.65	F	0.153	0.251	0.147	0.079	0.231	0.074	123
20 ^c	5.94	F	0.108	0.057	0.091	0.048	0.122	0.050	2436
21	5.26	F	0.063	0.182	0.394	0.671	0.055	0.059	343
22	32.85	F	0.112	0.162	0.227	0.386	0.054	0.077	181
23 ^c	11.05	M	0.153	0.092	0.081	0.057	0.144	0.080	2163.78
24	5.4	F	0.127	1.442	1.242	0.215	0.087	0.048	1047
25	8.92	F	0.073	2.199	2.136	1.947	0.047	0.056	1491
26	1	M	0.066	0.084	0.152	0.473	0.046	0.057	214
27	35.6	F	0.059	0.532	0.251	0.052	0.053	0.051	181.29
28 ^d	4.2	M	0.458	0.325	0.399	0.468	0.064	0.120	2187
29 ^d	4.39	F	1.221	0.908	1.910	1.982	0.241	0.098	2104
30 ^d	18.83	M	0.186	0.208	0.790	1.517	0.045	0.043	2832
31 ^d	2.4	F	0.286	0.137	0.171	0.145	0.048	0.053	304
32 ^d	27.39	F	0.355	0.740	0.680	0.450	0.130	0.120	545.5
33 ^d	3.31	M	1.982	3.900	3.850	1.210	0.140	1.350	2853
34 ^d	13.19	M	3.268	0.920	0.300	0.180	0.160	0.730	2477
Mean (SD)	15.4 (13.2)	44% M	0.46 (0.78)	0.59 (0.78)	0.8 (0.97)	0.59 (0.85)	0.1 (0.09)	0.15 (0.28)	1067.05 (863.06)

^aSpecific IgE levels are indicated as optical density at 492 nm. Optical density at 492 nm of 0.095 was used as cutoff value. Serum samples with higher optical density were considered as IgE positive and are indicated in bold.

^bMonosensitized to *Aedes aegypti*.

^cSensitized to *A. aegypti* and *Periplaneta americana*.

^dSerum samples from these patients were used for preparing the pool of 7 serum samples.

Table 2Clinical Characteristics of the Serum Samples Used in the Serum Pool^a

Patient no.	Diagnosis	Mosquito SPT	<i>Dermatophagoides pteronyssinus</i> SPT	<i>Blomia tropicalis</i> SPT	Cockroach SPT	Shrimp SPT
28	A, C	+	+	+	+	+
29	A	+	+	+	+	+
30	A, AD	+	+	+	–	+
31	R	+	+	+	+	+
32	C, AD	+	+	+	–	–
33	R	+	+	+	–	+
34	AD, R	+	+	+	–	+

Abbreviations: A, asthma; AD, atopic dermatitis; C, conjunctivitis; R, rhinitis; SPT, skin prick test.

^aPlus sign indicates positive test result; minus sign, negative test result.

Determination of Specific IgE

Plastic Microtiter, high-binding microtiter plates (Greiner Bio-One, Monroe, North Carolina) were coated overnight with 0.5 µg of extract per well. After washing 3 times with PBS-T (PBS, pH 7.4, and 0.25% Tween 20), nonspecific binding was blocked by incubation for 1 hour with PBS-T that contained 1% bovine serum albumin (BSA). After 3 additional washings, plates were incubated for 2 hours with individual serum samples, diluted 1:8 in PBS-T plus 1% BSA, then washed and incubated for 1 hour with 1:2,000 horseradish peroxidase-conjugated mouse anti-human IgE (Southern Biotech, Birmingham, Alabama). Finally, microplates were washed and incubated for 30 minutes with the substrate o-phenylenediamine dihydrochloride (Sigma-Aldrich, St Louis, Missouri). The reaction was stopped with 3.2% hydrochloride, and optical density was read at 492 nm. All the experiments were performed at 4°C in duplicate. Serum samples from 9 patients sensitized to pollens were used as negative controls. The mean of IgE levels from these samples +3 SDs were used as the cut off for positive IgE levels.

ELISA Inhibition Assays

Cross-reactivity studies were performed using a serum pool prepared by mixing equal volumes of 7 selected serum samples. All 7 patients had a positive SPT result with mosquito and mite extracts and detectable specific IgE against these allergens. Furthermore, 4 of these individuals also had positive SPT result and detectable specific IgE to cockroach and 5 to shrimp (Table 1 and Table 2). Microplates were coated with *A. aegypti* extract and blocked as described before. Subsequently, 50 µL of serially diluted extracts (*D. pteronyssinus*, *B. tropicalis*, *L. vannamei*, and *P. americana*) or recombinant tropomyosins (Aed a 10.0201, Der p 10, Blo t 10, Lit v 1, and Per a 7) (inhibitors) and 50 µL of 1:4 diluted serum pool were added to the wells. Microplates were incubated overnight at 4°C. The reaction was developed as described above. The positive control consisted of serum pool adsorbed with PBS-T that contained 1% BSA without inhibitor. *B. verrucosa* extract was used as negative control.

SDS-PAGE and Immunoblotting

Proteins from the mosquito extract were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions using a Mini-PROTEAN II apparatus (Bio-Rad, Hercules, California). Ten micrograms of extract were mixed with a buffer that contained β-mercaptoethanol and boiled for 5 minutes and then separated in 12% continuous polyacrylamide gels at 120 V. After separation, gels were stained with GelCode Blue stain reagent (Life Technologies, Carlsbad, California). Precision Plus Protein All Blue Standards (Bio-Rad) were used to determine the relative molecular weights of the electrophoresed components. Proteins were transferred onto a 0.45-µm nitrocellulose membrane using a Transblot Semydry Electrophoresis transfer cell (Bio-Rad) at 20 V for 30 minutes. Nonspecific binding was blocked with PBS-T

that contained 5% BSA, for 45 minutes. Membranes were washed 2 times with PBS-T, then incubated with serum pool diluted 1:10 in PBS-T that contained 1% BSA, for 2 hours. After 3 further washings with PBS-T, the membranes were incubated with 1:2,000 horseradish peroxidase-conjugated mouse anti-human IgE (Southern Biotech) for 1 hour and incubated with ECL Prime detection reagent (GE Healthcare Life Sciences, Buckinghamshire, England). Chemiluminescence was detected with a GeneGnome apparatus (Syngene, Cambridge, United Kingdom). Precision Plus Protein WesternC Standards (Bio-Rad), previously incubated with Precision Protein StrepTactin-HRP Conjugate (Bio-Rad), were used to determine the relative molecular weights.

Immunoblot Inhibition

SDS-PAGE (12% polyacrylamide, under reducing conditions) and immunoblotting were performed as described above, except that transferred nitrocellulose membranes were incubated with the serum pool, previously adsorbed with 100 µg/mL of the homologous, heterologous, and control inhibitors.

Excision and In-Gel Protein Digestion of Electrophoresed Bands

Bands of 14.1, 15, 17.9, and 54.3 kDa in Coomassie-stained gels of electrophoresed *A. aegypti* extract were excised manually, then deposited in 96-well plates and processed automatically in a Proteiner DP (Bruker Daltonics, Bremen, Germany). Tryptic digestion was performed as described by Schevchenko et al.,³⁴ with minor variations. Gel plugs were washed with 50 mM ammonium bicarbonate (ABC) and with acetonitrile (ACN) before reduction with 10 mM dithiothreitol in 25 mM ABC solution. Alkylation was performed with 55 mM iodoacetamide in 50 mM ABC. Gel pieces were rinsed and dried under a nitrogen stream. Proteomics Grade Trypsin (Sigma-Aldrich) at a final concentration of 16 ng/µL in 25% ACN/50 mM ABC solution was added for digesting at 37°C for 4 hours. The reaction was stopped with 50% CAN and 0.5% trifluoroacetic acid. The tryptic eluted peptides were dried by speed-vacuum centrifugation and dissolved in MALDI solution (30% ACN, 15% isopropanol, and 0.5% trifluoroacetic acid). An aliquot of each peptide mixture was deposited onto a 384-well OptiTOFTM Plate (SCIEX, Foster City, California) and allowed to dry at room temperature; then an aliquot of matrix solution (3 mg/mL of α-cyano-4-hydroxycinnamic acid in MALDI solution) was added and allowed to dry at room temperature.

MALDI Peptide Mass Fingerprinting, MS/MS Analysis, and Database Searching

Digested bands were characterized by MALDI-TOF/TOF mass spectrometry (MS+MS/MS) at Centro Nacional de Biotecnología, Madrid, Spain. For this analysis, samples were automatically acquired in an Abi 4800 MALDI TOF/TOF mass spectrometer (SCIEX, Foster City, California) in positive ion reflector mode (the ion acceleration voltage was 25 kV to MS acquisition and 2 kV to MS/MS) and spectra stored into the ABI 4000 Series Explorer Spot.

Peptide mass fingerprinting (PMF) and MS/MS fragment ion spectra were smoothed and corrected to zero baseline using routines embedded in ABI 4000 Series Explorer Software, version 3.6 (SCIEX, Foster City, California). Each PMF spectrum was internally calibrated with the mass signals of trypsin autolysis ions to reach a typical mass measurement accuracy of less than 25 ppm. Known trypsin and keratin mass signals, as well as potential sodium and potassium adducts (+21 Da and +39 Da), were removed from the peak list. To submit the combined PMF and MS/MS data to MASCOT software, version 2.5.10 (Matrix Science, London, United Kingdom), GPS Explorer software, version 4.9, was used, searching in the *A. aegypti* protein database from Uniprot-SwissProt repository (UKBsp-p7159_20150327; 17740 sequences; 8088037 residues). The confidence interval for protein identification was set to 95% or greater ($P < .05$), and only peptides with an individual ion score above the identity threshold were considered correctly identified.

BLASTp analysis was performed with the sequences of the characterized proteins to identify potential cross-reactive proteins in mites, cockroach, and shrimp.

Structural Modeling and Bioinformatics Analyses of the Amino Acid Sequence of Peptidyl-Prolyl Cis-trans Isomerase From *A. aegypti*

A 3-dimensional homology model of *A. aegypti* peptidyl-prolyl cis-trans isomerase (PPIase) was generated using the SWISS-MODEL workspace via the Expasy web server.³⁵ The proposed 3-dimensional structure of the protein was modelled using the PDB template 2biu.1A (human cyclophilin; sequence identity: 73.01%; z score: -1.28; GMQE: 0.95; QMEAN: 1.60). The amino acid sequence of PPIase from *A. aegypti* was compared with other allergenic cyclophilin sequences using the multiple alignment viewer MView (<http://www.ebi.ac.uk/Tools/msa/mview/>). The sequences of cyclophilins from mite (*D. farinae*), mosquitoes (*Aedes albopictus*, *Anopheles gambiae*, and *Culex tarsalis*), flies (*Musca domestica* and *Drosophila melanogaster*), shrimp (*L. vannamei*), cockroach (*Blattella germanica*), bee (*Apis mellifera*), and human (*Homo sapiens*) were used to map the conserved sequences on the surface of the 3-dimensional structure of the mosquito PPIase using the program ConSurf, which scores the amino acids according to their degree of conservation.³⁶

Results

Specific IgE to Mosquito, Mites, Cockroach, and Shrimp Extracts in Serum Samples From Allergic Patients

Twenty-seven serum samples (79.41%) were positive for *D. farinae*, 25 (73.53%) for *D. pteronyssinus* and *B. tropicalis*, 22 (64.7%) for *A. aegypti*, 10 (29.41%) for *P. americana*, and 8 (23.53%) for *L. vannamei*. Sensitization to at least one mite species was 82.35% (Table 1).

Tropomyosin Cross-reactivity Between Mosquito and Other Arthropods

Enzyme-linked immunosorbent assays (ELISAs) using *A. aegypti* on the solid phase revealed that at 50 $\mu\text{g/mL}$ *D. pteronyssinus*, *L. vannamei*, *B. tropicalis*, and *P. americana* inhibited the IgE reactivity in 96.6%, 95.4%, 84.4%, and 75.4%, respectively. The unrelated extract used as control, *B. verrucosa*, produced 10% inhibition (Fig 1A). When the recombinant tropomyosins were used as competitors with *A. aegypti* extract on the solid phase, maximum inhibitions of IgE reactivity of 55%, 51%, 50%, 41.5%, and 9.3% were obtained at the higher concentration of Per a 7, Der p 10, Blo t 10, Lit v 1, and Aed a 10, respectively (Fig 1B).

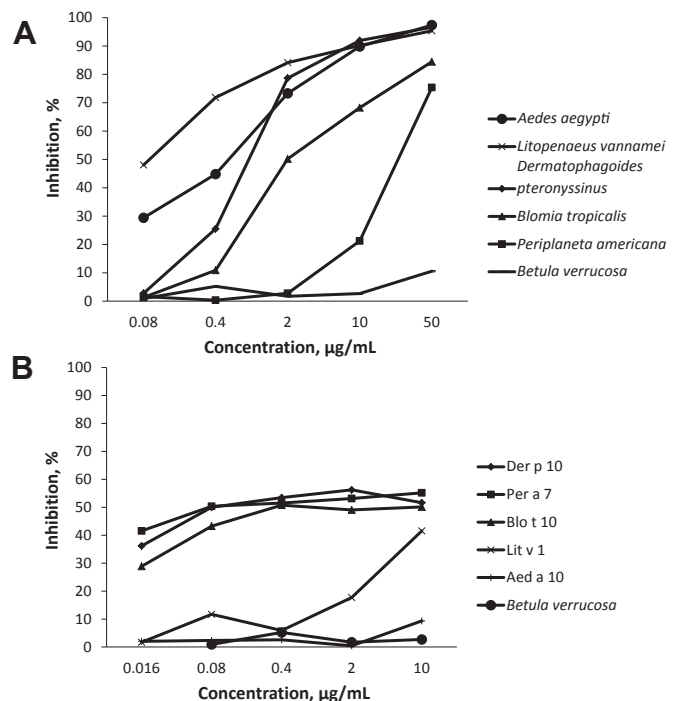


Figure 1. Enzyme-linked immunosorbent assay inhibition. Microplate wells coated with *Aedes aegypti* extract were incubated with pooled sera previously adsorbed with different concentrations of arthropod extracts (A) or recombinant tropomyosins (B) as inhibitors.

Involvement of Several IgE-Binding Components in the Cross-reactivity Between *A. aegypti* and Other Arthropods

Several bands between 10 and 150 kDa were detected in the *A. aegypti* extract. At least 11 bands (at 14.1, 15, 16.6, 17.9, 20.1, 28.3, 31.8, 46.6, 54.3, 74.2, and 124.1 kDa) reacted with specific IgE (Fig 2A). Homologous immunoblotting inhibition of IgE reactivity to *A. aegypti* extract with 100 $\mu\text{g/mL}$ of inhibitor revealed almost complete inhibition; no inhibition was observed with the *B. verrucosa* extract (Fig 2B).

In the case of using mite extract as an inhibitor, the inhibited bands were those of 74.2, 28.3, 17.9, 16.6, and 14.1 kDa. In the case of cockroach, cross-reactive bands were 124.1, 74.2, 54.3, 46.6, 28.3, 17.9, 16.6, and 15 kDa. In the case of shrimp, bands of 74.2, 28.3, 20.1, 17.9, and 16.6 kDa seem to be involved in cross-reactivity. Bands of 74.2, 28.3, 17.9, and 16.6 kDa were inhibited by all the inhibitors. The 31.8-kDa band of *A. aegypti* previously identified as tropomyosin³² had important reduction in the IgE reactivity when the serum pool was adsorbed with mite, shrimp, and cockroach extracts (Fig 2C).

Novel *A. aegypti* Allergens

The MALDI PMF and MS/MS analysis identified 4 proteins in the mosquito extract: the band of 14.1 kDa as odorant binding protein (OBP) (Uniprot Q1HRL7), the band of 15 kDa as mitochondrial cytochrome C (Uniprot Q1HRI9), the band of 17.9 kDa as PPIase (Uniprot Q1HRR9), and the band of 54.3 kDa as AAEL001668-PA (Uniprot Q17KK5), the product of the AAEL001668 gene with a hypothetical magnesium ion-binding function (Table 3). None of these proteins had been previously reported as mosquito allergens.

BLASTp analysis found homology of PPIase with Der f 29 allergen from *D. farinae*, and sequence alignment revealed 81.1% identity in the amino acid sequences (Fig 3). We further conducted a comparative structural analysis of PPIase with other related allergens.

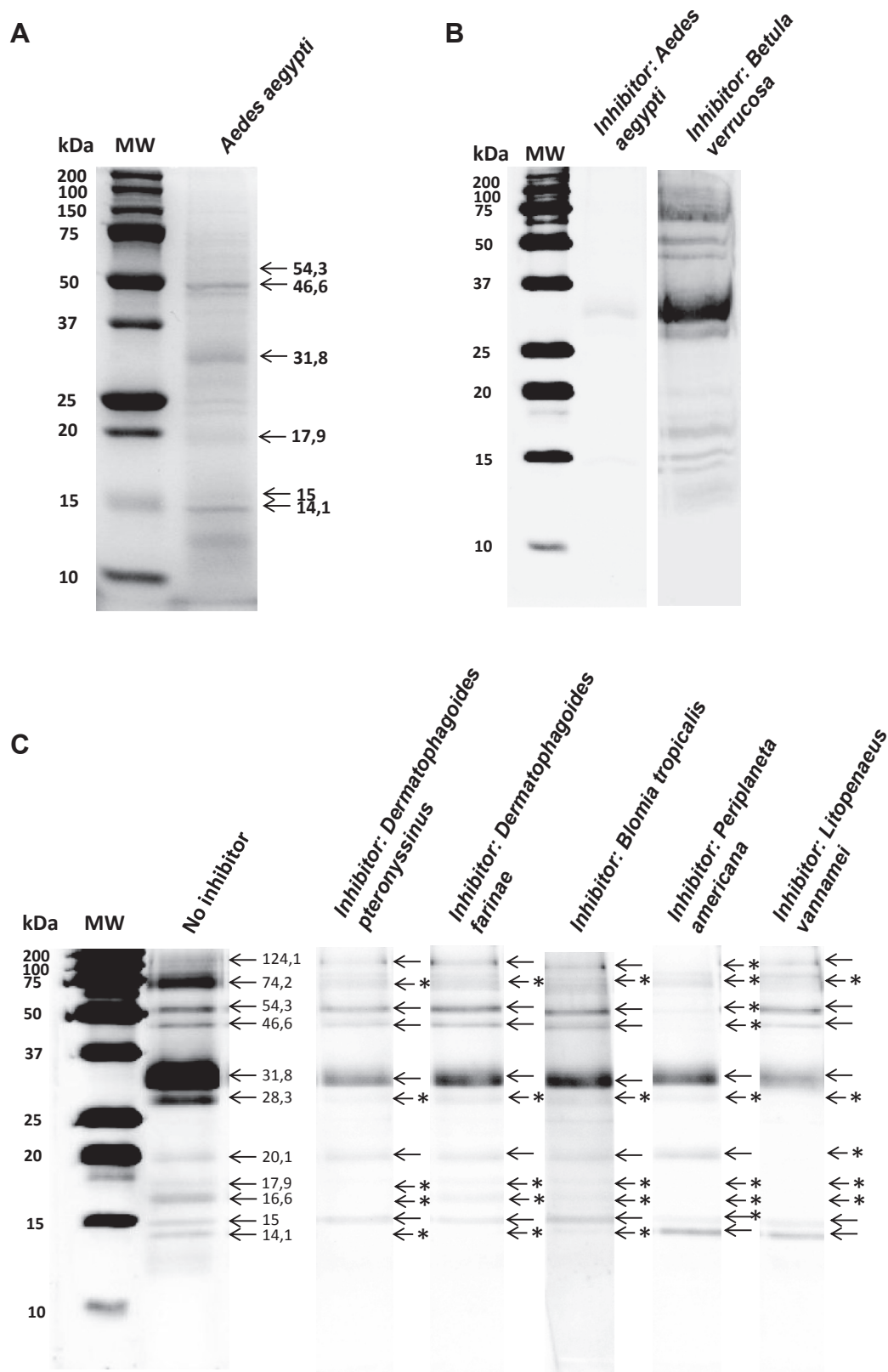


Figure 2. Coomassie blue–stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (A) and immunoblot inhibition of *Aedes aegypti* extract (B and C). Noninhibited bands are indicated with arrow and inhibited bands with arrow plus asterisk. MW, molecular weight.

Structural Analysis of PPLase From *A. aegypti*

The modeled PPLase from *A. aegypti* revealed a 3-dimensional folding consisting of a β -barrel of 8 antiparallel β -strands and 2 surrounding α -helices (Fig 4A,B). This folding is similar to other

cyclophilins. However, it differs in the β -barrel with 8 antiparallel β -strands instead of the typical β -barrel in cyclophilins, which contain 7. Multiple sequence alignment of PPLase from *A. aegypti* with cyclophilins reported as allergens (Fig 5A) reveals a high

Protein name	Uniprot	MASCOT score	E value	Coverage, %	MS (MS/MS)	Function	Theoretical MW, kDa	Experimental MW, kDa	Identity, %
Odorant-binding protein	Q1HRL7	185	5.60E-15	51	7 (2)	Odorant-binding	14.1	14.15	NF
Mitochondrial cytochrome C	Q1HRI9	140	1.80E-10	40	5 (1)	Ion-binding activity	15	15	NF
Peptidyl-prolyl cis-trans isomerase	Q1HRR9	410	1.80E-37	68	9 (5)	Isomerase rotamase	17.9	17.88	Der f 29 (81%)
AAEL001668-PA	Q17KK5	540	1.80E-50	63	21 (4)	Magnesium ion-binding	54.3	54	NF

degree of homology (>71%) with Der f 29 and with Rhi o 2 of *Rhizopus arrhizus*. A lower degree of homology (<22%) is observed when compared with cyclophilins, such as Sola l 5 from tomato (*Solanum lycopersicon*), Cat r 1 from *Catharanthus roseus*, Asp f 1 from *Aspergillus fumigatus*, Mala d 6 from *Malassezia dermatis*, and Bet v 7 from *Betula verrucosa*. Two highly conserved residues (⁹⁹SMANAGPNTNGSQFFI¹¹³ and ¹²¹WLDDRHVVFG¹³⁰ in the mosquito PPLase sequence) were previously identified in the cyclophilins allergen sequences and believed to be potential candidates for cross-reactivity.³⁷ These regions are indicated with arrows in Figure 5A. Region 1 is 100% conserved in the mosquito PPLase, and region 2 contains 2 substitutions (124 G-D and 125 K-R) on the amino acid sequence. Analysis of the surface residue conservation of the modeled PPLase with cyclophilins from mites, flies, shrimp, cockroach, bee, and human (Fig 5B) reveals a high degree of conservation in several surface areas, suggesting a strong possibility of cross-reactivity among cyclophilins, including *A. aegypti* PPLase.

In the present work, we found that *A. aegypti* cross-reacts with other arthropods and that several proteins are involved in this phenomenon. The clinical relevance of cross-reactivity is well documented for foods, pollens, and mites.³⁸ However, it is less clear for other allergen sources, such as mosquitoes. In the tropics, mites, cockroaches, and shrimp represent environmental risk factors for allergy. Therefore, and because of IgE cross-reactivity, coexposure to *A. aegypti* allergens may also have a clinical effect on patients with allergies. Similar effects have been proposed for *A. lumbricoides*, which seems to be an enhancer of the allergenic effects of mite allergens.^{39–41} The potential effect of mosquito allergens on allergic symptoms has not yet been defined, and the results of this study suggest that further research is clearly needed to clarify this issue.

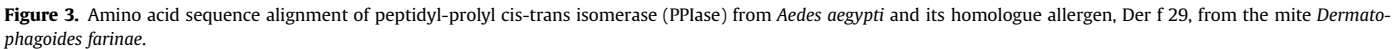
investigated, this frequency could result from the particular conditions of this Caribbean population, such as differences in climate, the increasing density of *A. aegypti*,⁴⁶ and the genetic background of the population. In addition, cross-reactivity with other arthropod allergens could also contribute to these findings. Mosquito specific allergens also seem to be involved in the sensitization process because 3 patients were monosensitized to *A. aegypti* and 3 were cosensitized to *A. aegypti* and *P. americana* (Table 1). Altogether, these data suggest that mosquitoes may represent an important allergen source for some patients in the Caribbean.

The tropics is one of the regions with the highest prevalence of *D pteronyssinus* and *B tropicalis* allergens in the environment. These 2 species are the most common in Cartagena, Colombia,⁴⁷ Puerto Rico,⁴⁸ San José de Costa Rica,⁴⁹ and Cuba.⁵⁰ These observations suggest that *D pteronyssinus* and *B tropicalis* are the primary sensitizers in the allergic population from Martinique.

In tropical regions, the allergenicity of *A. aegypti* and its capacity to cross-react with mite and other arthropod allergens must be considered when conducting routine allergen testing. An important task is to dissect whether the skin test reaction is the result of cross-reactivity or is a specific sensitization to several allergen sources. Whether the specificity of mosquito sensitization is biased by cosensitization to mites and other arthropods is a topic that must be further evaluated. Furthermore, the use of species-specific allergens may be helpful to solve this problem.

Immunoblots revealed 11 allergenic bands, ranging from 14 to 124.1 kDa, involved in the cross-reactivity between *A. aegypti* and the other arthropods. Furthermore, some bands of *A. aegypti* revealed cross-reactivity restricted to one arthropod. For example, the band of 14.1 kDa cross-reacts exclusively with mites, the bands of 124.1, 54.3, 46.6, and 15 kDa with cockroach, and the band of 20.1 kDa with shrimp.

Our results differ from those obtained by Wongkamchai et al.²⁶ Wongkamchai et al.²⁶ used serum samples from individuals with cutaneous allergic reactions, whereas our serum samples were derived from individuals with respiratory allergies. These authors identified only one IgE-reactive band at 42.9 kDa in a whole-body extract from *A. aegypti*, probably because of the different criteria used in the selection of the sera. Other studies have found that allergenic extracts prepared exclusively from saliva or salivary



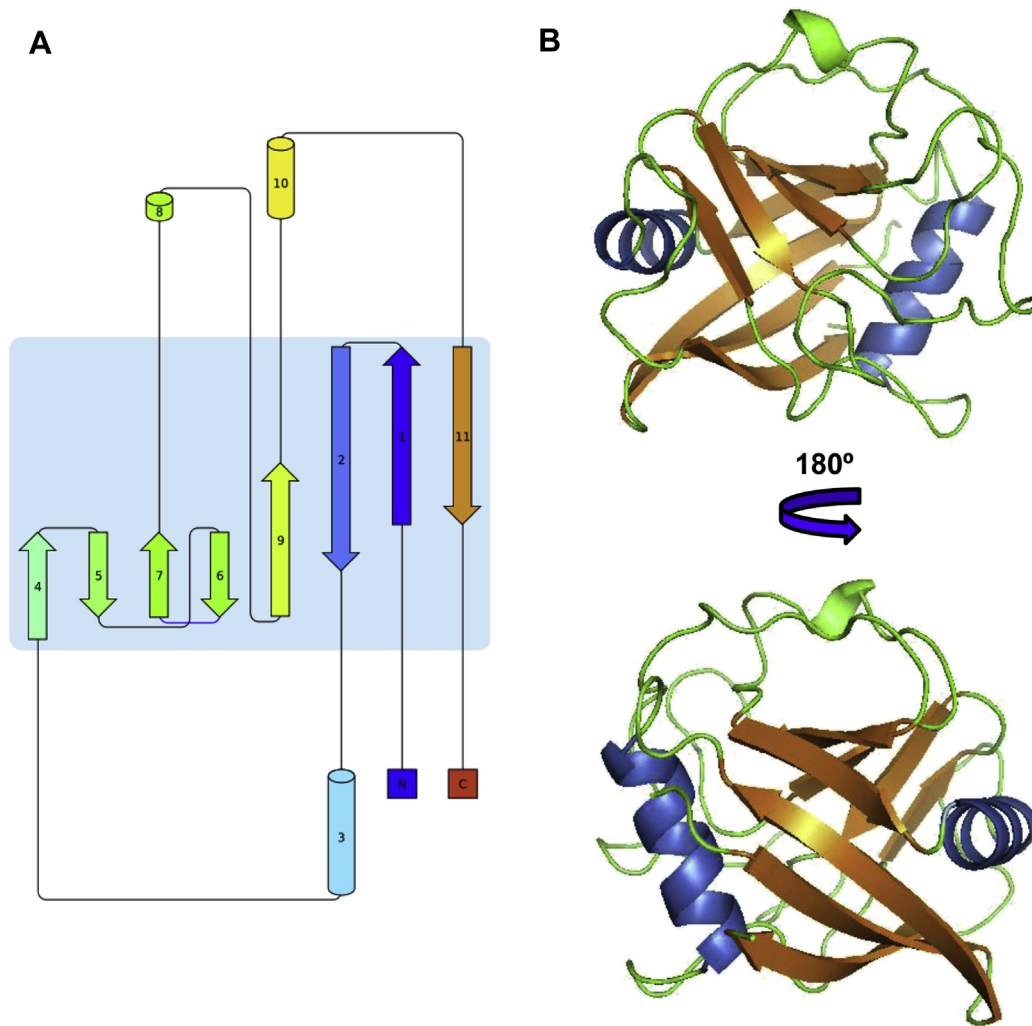


Figure 4. Structural characteristics of peptidyl-prolyl cis-trans isomerase from *Aedes aegypti*. (A) Topologic diagram showing the secondary structure elements. (B) Modeled 3-dimensional structure showing a β -barrel of 8 antiparallel β -strands and 2 surrounding α -helices.

glands contain the allergens involved in allergic skin reactions caused by mosquito bites.^{2,4,7}

Our SDS-PAGE and immunoblot results allowed the selection of 4 bands to further analysis by MS (Table 3). The identified proteins OBP, mitochondrial cytochrome C, and PPIase and the unknown protein AAEL001668-PA are novel IgE-binding proteins, and except for PPIase, no homologues of these molecules have been reported in mites, cockroach, or shrimp. From a taxonomic perspective, mosquitoes, mites, cockroach, and shrimp are related, and some degree of cross-reactivity is expected. This assumption correlated with the results obtained in the ELISAs. However, further homologous proteins shared by these species remain to be described.

In this work, we report the presence of an IgE-binding PPIase (band of 17.9 kDa) in *A. aegypti*, which seems to correspond to a potential panallergen. This band had cross-reactivity with all the inhibitors. In addition, we found a high sequence identity of this protein with the allergen Der f 29. Der f 29 reacted with IgE in 85.6% of the serum samples tested by immunoblotting⁵¹ and in 21% when analyzed by skin prick test in a Chinese mite allergic population.⁵² Several allergens homologous to PPIase or cyclophilin from plants or fungi have been characterized,^{53–55} suggesting that they may represent an important group of panallergen.

We further analyzed the potential cross-reactivity of the mosquito PPIase by a bioinformatics approach that allowed the

comparison of the primary, secondary, and tertiary structures of this protein with other members of the same protein family. The predicted secondary structure of the *A. aegypti* PPIase is similar to other cyclophilins^{37,56} that contain a β -barrel surrounded by 2 α -helices (Fig 4). Furthermore, its amino acid sequence is similar to sequences of allergenic cyclophilins from mites and molds (Fig 5A). These results suggest that *A. aegypti* PPIase may contain cross-reactive B-cell epitopes. Our analysis predicts that 2 regions on the amino acid sequence of cyclophilins, reported as putative IgE binding epitopes, are highly conserved in the sequence of the mosquito PPIase (Fig 5A). Furthermore, the surface of the modeled protein reveals several areas highly conserved in cyclophilins from diverse origins. All together, these results suggest that this protein could represent a novel panallergen.

The ELISA inhibition experiments revealed that the recombinant Aed a 10.0201, recombinant Lit v 1, recombinant Der p 10, recombinant Blo t 10, and Per a 7 inhibited less than 60% the IgE-binding to the *A. aegypti* extract. Furthermore, allergenic extracts of these arthropods produced higher inhibition (in some cases >95%). These results indicate that tropomyosin is not the only allergen involved in the cross-reactivity between *A. aegypti* and the other arthropods. *A. aegypti* tropomyosin inhibited less than 10% the IgE binding to the mosquito extract. This result may be explained by the apparent reduced IgE epitope representation in the variant Aed

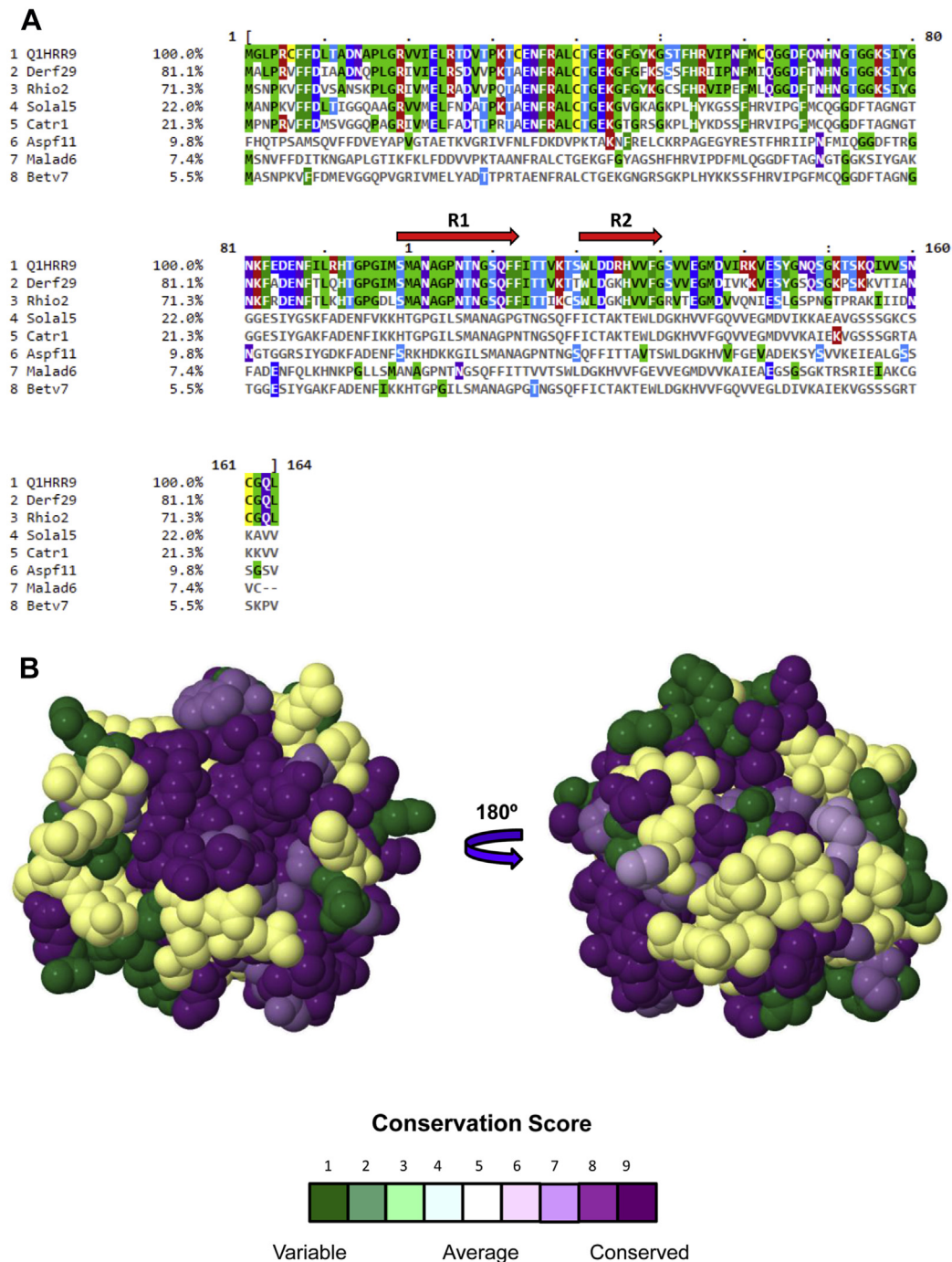


Figure 5. (A) Sequence alignment of *Aedes aegypti* peptidyl-prolyl cis-trans isomerase (PPIase) and cyclophilins from other allergenic sources, registered in allergome (www.allergome.org). (B) Sequence conservation mapped on the surface of *A. aegypti* PPIase using ConSurf.³⁶

a 10.0201, used in this study, compared with the Aed a 10.0101 variant.³² The use of this variant could result in higher inhibition.

These findings highlight the need to identify and characterize the complete set of cross-reactive and species-specific allergens from mosquitoes, mites, and other arthropods to better understand the molecular aspects of cross-reactivity and design improved strategies that may lead to better diagnosis and treatment of the allergic population, especially in the tropics where these allergenic sources are prevalent.

In conclusion, we found that *A. aegypti* cross-reacts with mites, shrimp, and cockroach and report that several proteins are potentially involved in this phenomenon, including 4 novel

allergens. Further studies are necessary to clarify the role of these molecules in the pathophysiology of allergies in the tropics, subtropics, and other places where mosquitoes are abundant.

References

- [1] Cantillo JF, Fernandez-Caldas E, Puerta L. Immunological aspects of the immune response induced by mosquito allergens. *Int Arch Allergy Immunol.* 2014;165:271–282.
- [2] Peng Z, Simons FE. Cross-reactivity of skin and serum specific IgE responses and allergen analysis for three mosquito species with worldwide distribution. *J Allergy Clin Immunol.* 1997;100:192–198.

- [3] Brummer-Korvenkontio H, Lappalainen P, Reunala T, Palosuo T. Detection of mosquito saliva-specific IgE and IgG4 antibodies by immunoblotting. *J Allergy Clin Immunol*. 1994;93:551–555.
- [4] Peng Z, Li H, Simons FE. Immunoblot analysis of salivary allergens in 10 mosquito species with worldwide distribution and the human IgE responses to these allergens. *J Allergy Clin Immunol*. 1998;101:498–505.
- [5] Kulthanan K, Wongkamchai S, Triwongwaranat D. Mosquito allergy: clinical features and natural course. *J Dermatol*. 2010;37:1025–1031.
- [6] Engler RJ. Mosquito bite pathogenesis in necrotic skin reactors. *Curr Opin Allergy Clin Immunol*. 2001;1:349–352.
- [7] Peng Z, Beckett AN, Engler RJ, Hoffman DR, Ott NL, Simons FE. Immune responses to mosquito saliva in 14 individuals with acute systemic allergic reactions to mosquito bites. *J Allergy Clin Immunol*. 2004;114:1189–1194.
- [8] Peng Z, Xu W, James AA, et al. Expression, purification, characterization and clinical relevance of rAed a 1—a 68-kDa recombinant mosquito *Aedes aegypti* salivary allergen. *Int Immunol*. 2001;13:1445–1452.
- [9] Peng Z, Xu W, Lam H, Cheng L, James AA, Simons FE. A new recombinant mosquito salivary allergen, rAed a 2: allergenicity, clinical relevance, and cross-reactivity. *Allergy*. 2006;61:485–490.
- [10] Jeon SH, Park JW, Lee BH. Characterization of human IgE and mouse IgG1 responses to allergens in three mosquito species by immunoblotting and ELISA. *Int Arch Allergy Immunol*. 2001;126:206–212.
- [11] Kang B, Chang JL. Allergenic impact of inhaled arthropod material. *Clin Rev Allergy*. 1985;3:363–375.
- [12] Pomes A. Cockroach and other inhalant insect allergens. *Clin Allergy Immunol*. 2008;21:183–200.
- [13] Kim CW, Hong CS. Allergy to miscellaneous household arthropods. *Protein Pept Lett*. 2007;14:982–991.
- [14] Puerta L, Fernandez-Caldas E, Lockey RF, Caraballo LR. Mite allergy in the tropics: sensitization to six domestic mite species in Cartagena, Colombia. *J Invest Allergol Clin Immunol*. 1993;3:198–204.
- [15] Puerta L, Fernandez-Caldas E, Lockey RF, Caraballo LR. Sensitization to *Chortoglyphus arcuatus* and *Aleuriglyphus ovatus* in *Dermatophagoides* spp. allergic individuals. *Clin Exp Allergy*. 1993;23:117–123.
- [16] Sohn MH, Kim KE. The cockroach and allergic diseases. *Allergy Asthma Immunol Res*. 2012;4:264–269.
- [17] Huss K, Adkinson NF Jr, Eggleston PA, Dawson C, Van Natta ML, Hamilton RG. House dust mite and cockroach exposure are strong risk factors for positive allergy skin test responses in the Childhood Asthma Management Program. *J Allergy Clin Immunol*. 2001;107:48–54.
- [18] Woo CK, Bahna SL. Not all shellfish “allergy” is allergy! *Clin Transl Allergy*. 2011;1:3.
- [19] Christophers SR. *Aedes aegypti* (L.) The Yellow Fever Mosquito. Its Life History, Bionomics and Structure. Cambridge, England: Cambridge University Press; 1960.
- [20] Soper F. *Ventures in World Health*. Washington, DC: Pan American Health Organization; 1977. Report 355.
- [21] Mattingly PF. Taxonomy of *Aedes aegypti* and related species. *Bull World Health Organ*. 1967;36:552–554.
- [22] Scott TW, Morrison AC, Lorenz LH, et al. Longitudinal studies of *Aedes aegypti* (Diptera: Culicidae) in Thailand and Puerto Rico: population dynamics. *J Med Entomol*. 2000;37:77–88.
- [23] Ponlawat A, Harrington LC. Blood feeding patterns of *Aedes aegypti* and *Aedes albopictus* in Thailand. *J Med Entomol*. 2005;42:844–849.
- [24] McBride CS, Baier F, Omondi AB, et al. Evolution of mosquito preference for humans linked to an odorant receptor. *Nature*. 2014;515:222–227.
- [25] Kausar MA, Vijayan VK, Bansal SK, Menon BK, Vermani M, Agarwal MK. Mosquitoes as sources of inhalant allergens: clinicoimmunologic and biochemical studies. *J Allergy Clin Immunol*. 2007;120:1219–1221.
- [26] Wongkamchai S, Khongtak P, Leemingsawat S, et al. Comparative identification of protein profiles and major allergens of saliva, salivary gland and whole body extracts of mosquito species in Thailand. *Asian Pac J Allergy Immunol*. 2010;28:162–169.
- [27] Smillie L. Structure and functions of tropomyosins from muscle and non-muscle sources. *Trends Biochem Sci*. 1979;4:5.
- [28] Asturias JA, Arilla MC, Gomez-Bayon N, Martinez A, Martinez J, Palacios R. Sequencing and high level expression in *Escherichia coli* of the tropomyosin allergen (Der p 10) from *Dermatophagoides pteronyssinus*. *Biochim Biophys Acta*. 1998;1397:27–30.
- [29] Asturias JA, Eraso E, Arilla MC, Gomez-Bayon N, Inacio F, Martinez A. Cloning, isolation, and IgE-binding properties of *Helix aspersa* (brown garden snail) tropomyosin. *Int Arch Allergy Immunol*. 2002;128:90–96.
- [30] Asturias JA, Gomez-Bayon N, Arilla MC, et al. Molecular characterization of American cockroach tropomyosin (*Periplaneta americana* allergen 7), a cross-reactive allergen. *J Immunol*. 1999;162:4342–4348.
- [31] Aki T, Kodama T, Fujikawa A, et al. Immunochemical characterization of recombinant and native tropomyosins as a new allergen from the house dust mite, *Dermatophagoides farinae*. *J Allergy Clin Immunol*. 1995;96:74–83.
- [32] Cantillo JF, Puerta L, Lafosse-Marín S, Subiza JL, Caraballo L, Fernandez-Caldas E. Identification and characterization of IgE-binding tropomyosins in *Aedes aegypti*. *Int Arch Allergy Immunol*. 2016;170:46–56.
- [33] Buendía E, Zakzuk J, Mercado D, Alvares A, Caraballo L. The IgE response to *Ascaris* molecular components is associated with clinical indicators of asthma severity. *World Allergy Organ J*. 2015;8:8.
- [34] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal Chem*. 1996;68:850–858.
- [35] Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics*. 2006;22:195–201.
- [36] Ashkenazy H, Abadi S, Martz E, et al. ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. *Nucleic Acids Res*. 2016;44:W344–W350.
- [37] Roy D, Ghosh D, Gupta-Bhattacharya S. Homology modeling of allergenic cyclophilins: IgE-binding site and structural basis of cross-reactivity. *Biochem Biophys Res Commun*. 2003;307:422–429.
- [38] Aalberse RC. Assessment of allergen cross-reactivity. *Clin Mol Allergy*. 2007;5:2.
- [39] Cooper PJ. Interactions between helminth parasites and allergy. *Curr Opin Allergy Clin Immunol*. 2009;9:29–37.
- [40] Ahumada V, Garcia E, Dennis R, et al. IgE responses to *Ascaris* and mite tropomyosins are risk factors for asthma. *Clin Exp Allergy*. 2015;45:1189–1200.
- [41] Suzuki M, Hara M, Ichikawa S, et al. Presensitization to *Ascaris* antigens promotes induction of mite-specific IgE upon mite antigen inhalation in mice. *Allergy Int*. 2016;65:44–51.
- [42] Bermanian MH, Korkinejad NA, Shirkhoda S, Nabavi M, Pourpak Z. Assessment of sensitization to insect aeroallergens among patients with allergic rhinitis in Yazd City, Iran. *Iran J Allergy Asthma Immunol*. 2012;11:6.
- [43] Sun BQ, Zheng PY, Zhang XW, Huang HM, Chen DH, Zeng GQ. Prevalence of allergen sensitization among patients with allergic diseases in Guangzhou, Southern China: a four-year observational study. *Multidiscip Respir Med*. 2014;9:2.
- [44] Fukutomi Y, Kawakami Y, Taniguchi M, et al. Allergenicity and cross-reactivity of booklice (*Liposcelis bostrychophila*): a common household insect pest in Japan. *Int Arch Allergy Immunol*. 2012;157:339–348.
- [45] Agarwal MK, Chaudhry S, Jhamb S, Gaur SN, Chauhan UP, Agarwal HC. Etiologic significance of mosquito (*Anopheles stephensi*) in respiratory allergy in India. *Ann Allergy*. 1991;67:598–602.
- [46] San Martín JL, Brathwaite O, Zambrano B, et al. The epidemiology of dengue in the Americas over the last three decades: a worrisome reality. *Am J Trop Med Hyg*. 2010;82:128–135.
- [47] Fernandez-Caldas E, Puerta L, Mercado D, Lockey RF, Caraballo LR. Mite fauna, Der p 1, Der f 1 and *Blomia tropicalis* allergen levels in a tropical environment. *Clin Exp Allergy*. 1993;23:292–297.
- [48] Montealegre F, Sepulveda A, Bayona M, Quinones C, Fernandez-Caldas E. Identification of the domestic mite fauna of Puerto Rico. *P R Health Sci J*. 1997;16:109–116.
- [49] Vargas M, Mairena H. House dust mites from metropolitan area of San Jose, Costa Rica. *J Acarol*. 1991;17:4.
- [50] Cuervo N, Dusbabek F, de la Cruz J, Abreu R. Acarids (Acarina: *Pyroglyphidae*, *Cheyletidae*, *Saproglyphidae* and *Glycyphagidae*) of house dust in Cuba. *Rev Cubana Med Trop*. 1983;35:83–103.
- [51] An S, Chen L, Long C, et al. *Dermatophagoides farinae* allergens diversity identification by proteomics. *Mol Cell Proteomics*. 2013;12:1818–1828.
- [52] Jiang C, Fan X, Li M, et al. Characterization of Der f 29, a new allergen from *Dermatophagoides farinae*. *Am J Transl Res*. 2015;7:1303–1313.
- [53] Fluckiger S, Fijten H, Whitley P, Blaser K, Cramer R. Cyclophilins, a new family of cross-reactive allergens. *Eur J Immunol*. 2002;32:10–17.
- [54] Cadot P, Diaz JF, Proost P, et al. Purification and characterization of an 18-kd allergen of birch (*Betula verrucosa*) pollen: identification as a cyclophilin. *J Allergy Clin Immunol*. 2000;105:286–291.
- [55] Marzban G, Herndl A, Kolarich D, et al. Identification of four IgE-reactive proteins in raspberry (*Rubus ideaeus* L.). *Mol Nutr Food Res*. 2008;52:1497–1506.
- [56] Ghosh D, Mueller GA, Schramm G, et al. Primary identification, biochemical characterization, and immunologic properties of the allergenic pollen cyclophilin cat R 1. *J Biol Chem*. 2014;289:21374–21385.