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TESIS DOCTORAL

Identificaction, cloning, purification and immunological characterization of unique and cross-reactive allergens of the mosquito species "Aedes aegypti"

Identificación, clonaje, purificación y caracterización inmunológica de alérgenos únicos y de reactividad cruzada del mosquito Aedes aegypti /

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PRESENTADA POR

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Submitted in partial fulfillment of the requirements for the degree of doctor

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To my brother Eduardo

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TABLE OF CONTENTS

I. RESUMEN

Aedes aegypti es una especie de mosquito originaria del continente africano. En los últimos 50 años ha experimentado una expansión acelerada hacia otros continentes; especialmente en áreas tropicales y subtropicales. Este insecto ha evolucionado con los seres humanos y ha aparecido una nueva subespecie, especializada en alimentarse a partir de sangre humana. Los mosquitos pertenecen al Phylum Arthropoda, que incluye varias especies como ácaros, gambas y langostinos, cucarachas y otros insectos inductores de reacciones alérgicas. La relación evolutiva y filogenética entre estas especies nos lleva a hipotetizar que los mosquitos contienen moléculas alergénicas que inducen respuestas alérgicas. La alta prevalencia de *A. aegypti*, y de otros mosquitos que viven en estrecho contacto con seres humanos, indica que la exposición a antígenos de este insecto ocurre frecuentemente.

Se ha propuesto que los mosquitos inducen respuestas alérgicas cutáneas, inducidas por antígenos salivares que se inyectan en la piel de los seres humanos, u otros animales, durante la picadura. Bajo este supuesto, el concepto de alergia al mosquito se acuñó para describir los signos y síntomas alérgicos que aparecen en la piel de individuos atópicos después de las picaduras de mosquitos. Teóricamente, estas reacciones sólo podrían ser producidas por mosquitos hembras, ya que los machos no necesitan sangre como fuente de nutrientes. Un grupo de investigadores liderados por el Dr. Peng ha dedicado sus esfuerzos a analizar estas reacciones e identificaron cuatro alérgenos derivados de la saliva. Como resultado de sus estudios, se demostró que la alergia a mosquitos sigue un mecanismo de sensibilización y desensibilización natural caracterizada por la producción simultánea de anticuerpos IgE y varias subclases de IgG.

En esta tesis, estudiamos la composición alergénica del mosquito *A. aegypti* y evaluamos su potencial alergénico y reactividad cruzada con otros alérgenos. En la mayoría de los experimentos, utilizamos sueros de pacientes alérgicos con asma y/o rinitis y sensibilizados a mosquitos y ácaros del polvo doméstico. Todos estos pacientes residían en áreas endémicas donde abundan los mosquitos. El uso de estos sueros en los ensayos de unión a IgE-específica nos ayudó en la identificación de los principales alérgenos potencialmente implicados en las reacciones alérgicas inducidas por *A. aegypti*.

Uno de los pasos iniciales que deben seguirse para responder a la pregunta de si los mosquitos son, o no son, fuentes de alérgenos, consiste en la identificación de proteínas de unión a IgE. Usamos dos enfoques para identificar alérgenos potenciales. Uno de ellos fue el análisis del Alergenoma de *A. aegypti*, utilizando herramientas de proteómica. Las proteínas totales de *A. aegypti* se extrajeron a partir de cuerpos del insecto y el espectro de proteínas se separó mediante electroforesis bidimensional. Los *spots* que unieron IgE específica se identificaron por immunoblot utilizando sueros individuales y una mezcla de sueros obtenidos de 15 pacientes alérgicos de la isla caribeña de Martinica. Los *spots* que resultaron positivos se localizaron en los geles 2-D teñidos con Coomassie y su identificación molecular se realizó por espectrometría de masas. Siguiendo este esquema, se identificaron diez proteínas diferentes y también se detectaron variantes, o isoformas, para algunas de ellas. Los 10 alérgenos identificados fueron: Heat shock cognate-70, Tropomiosina, Proteasa de aspártico lisosómica, Arginina o Creatina quinasa, Gliceraldehído-3-fosfato deshidrogenasa, Proteína de unión al calcio, Fosfoglicerato mutasa, proteína dependiente de voltaje con actividad de canal selectiva de aniones, proteína de unión a ATP y una proteína de función biológica desconocida. Curiosamente, encontramos que la mezcla de tres alérgenos parece ser suficiente para identificar más del 80% de los individuos sensibilizados a *A. aegypti*.

En un segundo enfoque, la tropomiosina natural de *A. aegypti* se purificó mediante una combinación de cromatografía de exclusión por tamaño y de intercambio iónico. Estas proteínas purificadas se seleccionaron utilizando experimentos de unión a IgE-específica en sueros de individuos alérgicos sensibilizados a las tropomiosinas de ácaro y gamba. El análisis de las fracciones purificadas mostró que en *A. aegypti*, la tropomiosina consiste en una mezcla de al menos 4 versiones (isoformas y variantes). Dos de estas versiones, las tropomiosinas con códigos UNIPROT: Q17H75 y Q17H80, se encontraron en mayores cantidades relativas. El análisis de la secuencia de

aminoácidos de estas tropomiosinas, y los experimentos de inhibición, indicaron que la variante Q17H75 tenía un mayor grado de homología con otras tropomiosinas y mayor conservación de epítopos putativos de unión a IgE, que la variante Q17H80, lo que sugiere que Q17H75 es más alergénica y de mayor reactividad cruzada.

Como resultado de estos experimentos, se registraron seis nuevos alérgenos de mosquitos en la base de datos de alérgenos: Aed a 5 (proteína sarcoplásmica de unión Ca^{+}), Aed a 6 (Porina 3), Aed a 7 (proteína de función biológica no definida), Aed a 8 (Heat shock cognate-70), dos variantes de tropomiosina (Aed a 10.0101 y Aed a 10.0201) y Aed a 11 (proteasa de aspártico lisosómica).

Con el fin de estudiar más a fondo el papel de Aed a 10.0101, Aed a 10.0201 y Aed a 8 en la sensibilización a mosquitos, las versiones recombinantes se expresaron en *E. coli*. rAed a 10.0101 se clonó en el vector de expresión pCOLDIV y se expresó bajo choque térmico a 15º C . rAed a 10.0201 y rAed a 8 se clonaron en el vector de expresión pET14b(+) y se expresaron bajo la estimulación con IPTG. Las proteínas recombinantes se purificaron por afinidad y se determinaron sus propiedades fisicoquímicas. rAed a 8, rAed a 10.0101 y rAed a 10.0201 mostraron actividad de unión a IgE e IgG e inhibieron la reactividad de anticuerpos contra su homólogo natural contenida en el extracto de mosquito. Igualmente mostraron capacidad de activación de basófilos e indujeron la proliferación de esplenocitos obtenidos de ratones inmunizados con estas moléculas. Además, rAed a 10.0101 y rAed a 10.0201 presentaron espectros de dicroísmo circular típicos de proteínas α-helicoidales y similares al espectro de rDer p 10. Estos resultados demostraron que los alérgenos recombinantes son similares a los alérgenos nativos y que cumplen con los requisitos inmunológicos y bioquímicos necesarios para ser utilizados de una manera similar al nativo.

El estudio de la respuesta inmune inducida por rAed a 8 reveló que después del protocolo de inmunización, los ratones BALB/c produjeron niveles bajos de IgE y altos niveles de varios subtipos de IgG, especialmente IgG2a, que ha sido propuesto como indicador de una inmunoterapia exitosa y como

anticuerpo bloqueador que inhibe la reactividad de la IgE con el alérgeno. Dado que el contenido de endotoxina de la muestra utilizada en la inmunización fue muy bajo, estos resultados sugieren que rAed a 8 tiene propiedades inmuno-reguladoras. Otros estudios con antígenos salivares utilizados en la inmunización de ratones mostraron resultados similares. La historia natural de la alergia a la picadura de mosquitos se caracteriza por la producción de subclases de IgG y conduce a la desensibilización de los individuos afectados. Las propiedades inmuno-reguladoras de rAed a 8, y otros componentes del mosquito, se deben seguir estudiando.

Además de estos descubrimientos, algunos estudios han sugerido que los mosquitos y otros insectos podrían inducir respuestas alérgicas por alérgenos no-salivares. Estas sugerencias se han basado en los siguientes puntos: 1) Los individuos alérgicos de diferentes países que sufren de asma y/o rinitis alérgica están sensibilizados frente los antígenos de los mosquitos; 2) El detritus del mosquito permanece suspendido en el aire y está presente en el polvo doméstico y 3) El análisis del genoma de *A. aegypti* indica que varios alérgenos, homólogos a los presentes en otras especies de Artrópodos, son expresados por este insecto.

Basados en estas observaciones, planteamos la hipótesis de que *A. aegypti* contiene proteínas que inducen diferentes tipos de respuestas alérgicas en individuos atópicos y que algunos de esos alérgenos presentan reacción cruzada con alérgenos de otras especies de artrópodos. Varios estudios han sugerido que las proteínas de los mosquitos tienen reactividad cruzada con alérgenos derivados de artrópodos. Inicialmente, estudiamos la reactividad cruzada entre extractos de *A. aegypti*, *Dermatophagoides pteronyssinus, Blomia tropicalis, D. farinae, Periplaneta americana* y *Litopenaeus vannamei*. Nuestros resultados demuestran que el mosquito tiene alta reactividad cruzada con el camarón, seguido por los ácaros y la cucaracha. Utilizando un grupo de 34 sueros de pacientes con alergias respiratorias, encontramos que el 64,7% de los individuos alérgicos estaban sensibilizados a *A. aegypti* y que esta prevalencia era similar a la prevalencia de sensibilización a ácaros y más alta que la prevalencia de sensibilización a gambas y cucarachas. Además, el 9% de los pacientes estaban mono-sensibilizados a mosquitos. Con el fin de

identificar los componentes de reactividad cruzada en el extracto de mosquito, se realizó un experimento de inhibición del immunoblot. Identificamos más de 10 bandas de reactividad cruzada y cuatro de ellas se caracterizaron por espectrometría de masas. Las bandas corresponden a las proteínas: proteína de unión a odorante, citocromo C mitocondrial, ciclofilina (PPIasa) y una proteína de función biológica desconocida "AAEL001668-PA". Curiosamente, encontramos que la tropomiosina representa aproximadamente el 40% de la reactividad a IgE, lo que significa que este no es el alérgeno más importante involucrado en la sensibilización a mosquitos.

La tropomiosina es un pan-alérgeno expresado por varias especies diferentes y responsable en gran medida de la reactividad cruzada entre los artrópodos. Se analizó la reactividad cruzada entre las tropomiosinas del ácaro (rDer p 10) y de *A. aegypti* (rAed a 10.0101 y rAed a 10.0201), desde un punto de vista molecular y funcional. Se encontró que rDer p 10 y rAed a 10.0101 presentaron mayor capacidad inhibitoria de la unión a IgE en comparación con rAed a 10.0201 utilizando el suero de los individuos alérgicos al ácaro del polvo doméstico. Además, la reactividad cruzada entre rDer p 10 y las tropomiosinas derivadas de mosquito indujo la activación de basófilos re-sensibilizados con sueros de pacientes que contenían altos niveles de IgE-específica contra rDer p 10.

rAed a 10.0101 y rAed a 10.0201 se utilizaron para inmunizar ratones BALB/c y se observó que la IgG1 específica producida por estos animales presentó reactividad cruzada con rDer p 10, las tropomiosinas de mosquito y con sus homólogos naturales presentes en el extracto. Los sueros de los ratones también inhibieron la activación de basófilos sensibilizados con anticuerpos específicos de rDer p10. También se estudió la reactividad cruzada a nivel de células T. Los esplenocitos de los ratones inmunizados con rAed a 10.0101 proliferaron tras la estimulación con rAed a 10.0101 y en menor grado a rAed a 10.0201 y rDer p 10. En contraste, los esplenocitos de ratones inmunizados con rAed a 10.0201 experimentaron una proliferación similar tras la estimulación con las tres tropomiosinas. El mapeo de epítopos utilizando 28 péptidos solapantes que cubrían la secuencia completa de Aed a 10.0101 reveló cinco regiones de aminoácidos que contienen epítopos T implicadas en esta reactividad cruzada.

En conclusión, hemos demostrado que *A. aegypti* es una fuente importante de alérgenos que pueden jugar un papel en enfermedades alérgicas, especialmente en áreas tropicales y subtropicales. Los alérgenos de los mosquitos pueden participar potencialmente en el inicio (fase de sensibilización) y en la exacerbación de las respuestas alérgicas. Varios alérgenos están involucrados en estos fenómenos y algunos de ellos tienen reactividad cruzada con alérgenos derivados de artrópodos. Nuestros estudios con tropomiosinas revelaron que la reactividad cruzada puede ocurrir a nivel molecular y funcional, involucrando anticuerpos, basófilos y linfocitos T. Los alérgenos de los mosquitos pueden producirse como moléculas recombinantes con propiedades biológicas y estructurales similares a las de los alérgenos naturales. Los estudios de la respuesta inmunológica inducida en ratones por rAed a 8 indicaron que este alérgeno puede tener un papel inmuno-regulador intrínseco. Más estudios epidemiológicos y utilizando los alérgenos purificados son necesarios para entender la alergia a mosquito.

II. SUMMARY

Aedes aegypti is a mosquito species originary from the African continent. In the last 50 years it has experienced an accelerated spread into other continents, especially to tropical and subtropical areas. This insect has co-evolved with humans and a new sub-species, specialized on feeding on human blood, has appeared. Mosquitoes are part of the Phylum Arthropoda, which includes several species like mites, cockroaches, shrimps and other insects that induce allergic responses. The evolutionary relationship among these species leads us to the hypothesis that mosquitoes would also contain allergenic molecules which induce allergic responses in humans. The high prevalence of *A. aegypti* and other mosquitoes living in close contact with humans, indicate that exposure to their antigens occurs frequently.

It is well established that mosquitoes induce cutaneous allergic responses through salivary antigens that are injected in the skin of humans, or other animals, during blood feeding. Under this assumption, the concept of mosquito allergy was coined to describe the allergic signs and symptoms that appear in the skin after mosquito bites in atopic individuals. Theoretically, these reactions could only be produced by female mosquitoes, since males don’t need blood as their source of nutrients. A group of researchers directed by Dr. Peng dedicated their efforts to analyse these reactions and identified 4 saliva-derived allergens. As a result of their studies, it was demonstrated that mosquito allergy follows a mechanism of natural sensitization and desensitization characterized by the simultaneous production of IgE and IgG antibody subclasses.

In this thesis, we studied the allergenic composition of the mosquito *A. aegypti* and evaluated their allergenic potential and cross-reactivity with other insect and mite allergens. In the majority of the experiments, we used sera from allergic patients with asthma and/or rhinitis and sensitized to mosquitoes and house dust mites. All these patients resided in areas where mosquitoes are abundant. The use of these sera in the specific IgE-binding assays helped us in the identification of the main allergens potentially involved in the allergic reactions.

One of the initial steps that we followed to answer the question of whether, or not, mosquitoes are the source of allergens consisted in the identification of IgE-binding proteins. We used two approaches to identify potential allergens. One of then was the analysis of the *A. aegypti* Allergenome, using proteomic tools. Total *A. aegypti* proteins were extracted from mosquito bodies and the protein spectrum separated by 2-dimensional electrophoresis. The IgE-binding spots were identified by immunoblotting using pooled and individual sera from 15 allergic patients from the Caribbean island of Martinique. The spots that resulted positive were located on the Coomassie stained 2-D gels and their identity determined by mass spectrometry. Following this scheme, ten different proteins were identified and several variants or isoforms were also detected. The 10 identified allergens were: Heat shock cognate-70, Tropomyosin, Lysosomal aspartic protease, Arginine or Creatine kinase, Glyceraldehyde-3-phosphate dehydrogenase, Calcium-binding protein, Phosphoglycerate mutase, Voltage-dependent anion-selective channel activity protein, ATP-binding protein and an Unknown protein. Interestingly, we found that the mixture of three allergens seems to identify more than 80% of *A. aegypti* sensitized individuals.

In a second approach, the natural tropomyosin from *A. aegypti* was purified by a combination of size exclusion and ionic exchange chromatography. These purified proteins were screened by means of specific IgE-binding experiments using the sera from allergic individuals sensitized to mite and shrimp tropomyosins. The analysis of the purified fractions showed that in *A. aegypti*, tropomyosin consists of a mixture of at least 4 versions (isoforms and variants). Two of these versions, with UNIPROT codes: Q17H75 and Q17H80, were found in higher concentrations. Amino acid sequence analysis and inhibition experiments indicated that tropomyosin Q17H75 had a higher degree of homology with other tropomyosins and preserved more putative IgE-binding epitopes than Q17H80, suggesting that Q17H75 is more allergenic and cross-reactive.

As a result of these experiments, 6 novel mosquito allergens were registered in the allergen database, namely, Aed a 5 (Sarcoplasmic Ca+ (EF-hand) binding protein), Aed a 6 (Porin 3), Aed a 7(Undefined protein), Aed a 8 (Heat shock cognate-70), two variants of tropomyosin (Aed a 10.0101 and Aed a10.0201) and Aed a 11 (Lysosomal aspartic protein).

In order to further study the role of these and other allergens in mosquito sensitization, the recombinant versions were expressed in *E. coli*. rAed a 10.0101 was cloned in the expression vector pCOLDIV and expressed under cold-shock in *E. coli*. rAed a 10.0201 and rAed a 8 were cloned in the expression vector pET14b(+) and expressed under stimulation with IPTG. The recombinant proteins were purified by affinity and their physicochemical properties determined. rAed a 8, rAed a 10.0101 and rAed a 10.0201 showed IgE and IgG-binding activity and inhibited the antibody reactivity of the natural counterpart contained in the mosquito extracts. They also showed basophil activation capacity and induced the proliferation of splenocytes obtained from mice immunized with these molecules. Furthermore, rAed a 10.0101 and rAed a 10.0201 presented circular dichroism spectra typical for α-helical proteins and similar to the rDer p 10 spectrum. These results demonstrated that the recombinant allergens are similar to the native allergens and that they fulfil the immunological and biochemical requisites needed to be used in allergy research.

The study of the immune response induced by rAed a 8 revealed that after immunization, the BALB/c mice produced low levels of IgE and high levels of IgG subclasses, specially IgG2a, which has been postulated as an indicator of successful immunotherapy and a blocking antibody that inhibit the reactivity of the IgE with the allergen. Since the endotoxin content of the sample used in the immunization was very low, these results strongly suggest that rAed a 8 has immune-regulatory properties. Other studies with salivary antigens used for the immunization of mice showed similar results. Furthermore, the natural history of mosquito bite allergy is characterized by the production of IgG subclasses and leads to the desensitization of the affected individuals. The immune-regulatory properties of rAed a 8, and other mosquito components, should be further studied.

In addition to these discoveries, some studies have suggested that mosquitoes, and other insects, may potentially induce different allergic responses by allergens not restricted to the saliva. These suggestions are based on the following points: 1) Allergic individuals from different countries who suffer from asthma and/or allergic rhinitis have specific IgE against mosquito antigens; 2) Mosquito detritus remains suspended in the air and is present in settled house dust and 3) The analysis of *A. aegypti* genome indicates that several allergens, homologues to those present in other Arthropod species, are expressed by this insect. Based on these facts, we hypothesized that *Aedes aegypti* contains proteins which induce different forms of allergic responses in atopic individuals, and that some of those allergens cross-react with allergens from other arthropod species.

Several studies have suggested that mosquito proteins cross-react with arthropod-derived allergens. Initially, the cross-reactivity among *A. aegypti, Dermatophagoides pteronyssinus, Blomia tropicalis, D. farinae, Periplaneta americana* and *Litopenaeus vannamei* was studied with whole extracts prepared from these sources. Our results showed that mosquito strongly cross-reacts with shrimp, followed by mites and cockroach. Using a group of 34 sera from patients with respiratory allergies, we found that 64.7% of the allergic individuals were sensitized to *A. aegypti* and that this prevalence was similar to the prevalence of IgE-sensitization to mites and higher than the prevalence of sensitization to shrimp and cockroach. Furthermore, 9% of the patients were mono-sensitized to mosquito. In order to identify the cross-reactive components in the mosquito extract, an immunoblotting inhibition experiment was performed. More than 10 cross-reactive bands were identified and 4 of them further characterized by Mass Spectrometry. The bands corresponded to the proteins: Odorant Binding Protein, Mitochondrial cytochrome C, Cyclophilin (PPIase) and an unknown protein “AAEL001668-PA”. Interestingly, we found that tropomyosin accounts for approximately 40% of the IgE reactivity, which means that it is not the most important allergen involved in mosquito IgE-sensitization.

Tropomyosin is a pan-allergen expressed by several different species and responsible to a great extent for the cross-reactivity among arthropods. We analysed the cross-reactivity between mite tropomyosin (rDer p 10) and *A.*

aegypti tropomyosins (rAed a 10.0101 and rAed a 10.0201) from a molecular and functional point of view. We found that rDer p 10 and rAed a 10.0101 presented a greater inhibitory capacity of the IgE-binding than rAed a 10.0201 using the sera from house dust mite allergic individuals. In addition, the cross-reactivity of rDer p 10 and the mosquito-derived tropomyosins induced the activation of stripped basophils sensitized with sera of patients containing high levels of Der p 10-specific IgE.

rAed a 10.0101 and rAed a 10.0201 were used to immunize BALB/c mice and we observed that the specific IgG1 produced by the animals cross-reacted with rDer p 10, the mosquito tropomyosins and with their natural counterparts in the extract. The murine sera also inhibited the activation of basophils sensitized with Der p 10-specific antibodies.

We also studied the cross-reactivity at the T-cell level. Splenocytes from mice immunized with rAed a 10.0101 showed strong proliferation upon stimulation with rAed a 10.0101 and to a lesser extent with rAed a 10.0201 and rDer p 10. In contrast, splenocytes from mice immunized with rAed a 10.0201 experienced similar proliferation indexes upon stimulation with the 3 tropomyosins. Epitope mapping using 28 overlapping peptides covering the whole sequence of Aed a 10.0101 revealed that 5 T cell epitope-containing regions are involved in this cross-reactivity.

In conclusion, we have demonstrated that the mosquito species *A. aegypti* is an important source of allergens that may play a role in allergic diseases, especially in tropical and subtropical areas. The mosquito allergens may potentially participate in the onset and exacerbation of the allergic responses. Several allergens are involved in these phenomena and some of them cross-react with arthropod-derived allergens. Our studies with tropomyosins reveal that cross-reactivity may occur at the molecular and functional levels, involving antibodies, basophils and T lymphocytes. Mosquito allergens can be produced as recombinant molecules that mimic the natural allergen. Studies of the immune response elicited by rAed a 8 in mice indicate that this allergen may have an intrinsic immune-regulatory role. Further analysis with the purified allergens and epidemiological studies are necessary.

III. INTRODUCTION

A. The role of arthropods in the allergic responses

Allergic diseases, like asthma, rhinitis, atopic dermatitis and papular urticaria are health problems affecting a large number of individuals, who usually face a worsening of the allergic manifestations in several moments of their lives. It has been estimated that 25% of the global population is affected by an allergic disease and this percentage is raising (1).

Arthropods encompass a great diversity of animal taxa known from the Cambrian to the present days. Arthropod evolutionary relationship is still a matter of debate. It has been widely accepted that they include four living groups: Myriapods, Chelicerates (which include the class Arachnida that contains mite species like *Dermatophagoides pteronyssinus* and *Blomia tropicalis*), Insects (which includes cockroach and mosquito species like *Periplaneta americana* and *Aedes aegypti*, respectively) and Crustaceans (which include shrimp species like *Litopenaeus vannamei*). These groups are known collectively as Euarthropoda. Furthermore, two other living groups, the soft bodied Onychophorans (velvet worms) and the microscopic tardigrades (water bears) are close relatives of the Euarthropods and together are referred to as Pan-arthropoda (2).

However, the analysis of several groups of genes that involve not only the nuclear, ribosomal and mitochondrial genes, but also expressed sequence tags (ESTs) from cDNA libraries, revealed an arthropod phylogeny tree as shown in Figure 1.

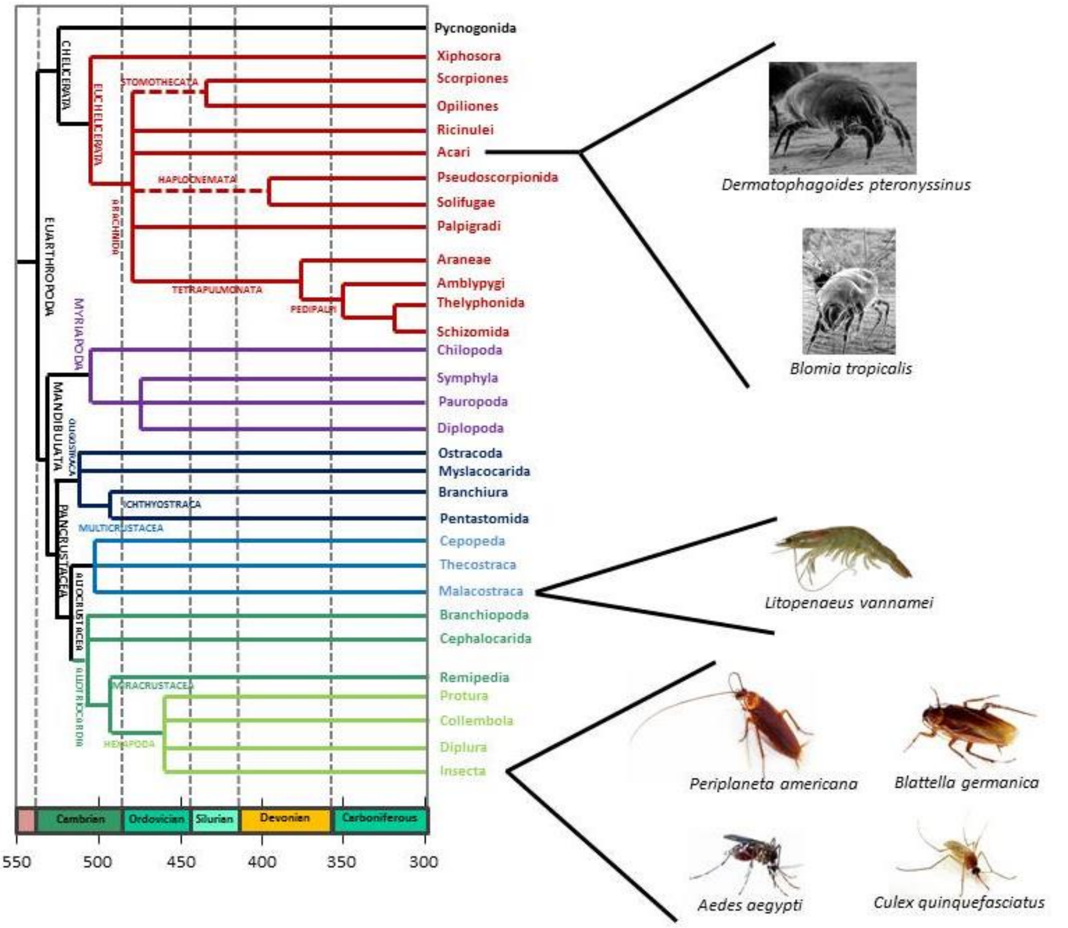

Figure 1. Phylogenetic framework for extant Arthropoda. Dashed lines within Arachnida indicate morphological grouping. Some arthropod species as sources of allergens in Acari, Malacostraca and Insecta are shown. Modified from (3).

Arthropods are similar to another Phylum, the annelid worms, which are also characterized by segmentation and the structure of their nervous and blood vascular systems. Furthermore, in 1997 the concept of Ecdysozoa, a clade consisting of pan-arthropods and a group of lesser-known worms named the Cycloneuralia, comprising the Priapulids, Kinorhynchs, Loriciferans, Nematodes (e.g. *Ascaris lumbricoides*) and Nematomorphs was introduced (4).

Arthropods are among the most important sources of allergens, including 3 major groups that are involved in allergic diseases. Insects and Arachnids are common sources of inhalant allergens, while Crustaceans are sources of food allergens. Mites and cockroaches are sources of indoor allergens and members of the Arachnid and Insect classes, and play an important role in respiratory allergies, like asthma. Human exposure to these two arthropods is perennial and the airborne particles which appear for short periods of time upon disturbance, carry their allergens which are inhaled and may induce sensitization and allergic symptoms.

House dust mites (HDM) are ubiquitous in human habitats and one of the most frequent sources of indoor allergens responsible for allergic sensitization and reactions in the upper and lower respiratory tract (5, 6). It is estimated that 1% to 2% of the world’s population might be affected by HDM allergy, which is equivalent to 65 to 130 million of individuals (7). Although HDM are consistently found in most places with a high ambient humidity, prevalence of allergic responses induced by these species is variable. According to the European Community Respiratory Health Survey I, the mean prevalence of sensitization among allergic patients is 21.7% (8). In Latin America HDM are common in Peru, Brazil, Venezuela, Colombia, Costa Rica, Puerto Rico and other countries (9-15), where the prevalence of sensitization ranges from 60% to 97% (16-18). More than 80% of prevalence is found in the pediatric population in Taiwan (19).

Cockroaches are other important indoor allergen sources which include more than 4,600 species and are particularly relevant in the USA and other countries. The best studied cockroach species are the German cockroach (*Blattella germanica*), which dominates in the US, as well as the American cockroach (*Periplaneta americana*) and the oriental or common cockroach (*Blatta orientalis*). A strong association between cockroach allergy, allergic rhinitis, and asthma has been demonstrated (20-22). In the USA, exposure and sensitization to cockroach allergens are associated with increased asthma morbidity in children (21, 22).

Consumption of seafood, including shrimps, is associated with the development of allergic reactions. These reactions might be produced by the ingestion of food containing the allergens, but also by contact, or inhalation (23, 24). Allergic sensitization to shrimp is variable and depends on the level of consumption and exposure. In a population of 17,366 adult allergic patients from different regions of Europe covering the Northern Maritime (Reykjavik), Northern (Vilnius), Central (Lodz), Balkan (Sofia), Alpine (Zurich), Mediterranean (Athens, Madrid) and Atlantic seaboard (Utrecht) areas, 4.67% were sensitized to shrimps (25). In a study with 8,203 individuals in the USA it was found that 0.9% to 1.2% of 20 to 59 years old individuals had anti-shrimp specific IgE; this prevalence decreased to 0.7% in the population having >60 years of age (26).

Arthropods are evolutionary related and share homologous proteins that play a key role in the frequently described allergenic cross-reactivity. Since cockroaches and mosquitoes are members of the class Insecta, a high degree of cross-reactivity between them is expected. In turn, the proximity of these animals with shrimps, followed by mite species, suggests some degree of cross-reactivity (See figure 1).

B. Taxonomical classification of Mosquitoes

Mosquitoes are arthropods that belong to the class Insecta, order Diptera and members of a family of the nematocerid flies Culicidae (from the Latin culex, genitive culicis, meaning ‘midge’ or ‘gnat’). The word ‘mosquito’ (formed by ‘mosca’ and the diminutive ‘ito’) is from the Spanish or Portuguese language for ‘little fly’. Mosquitoes resemble crane flies (family Tipulidae) and chironomid flies (family Chironomidae). The females of many species of mosquitoes are blood-eating pests and dangerous vectors of disease. However, members of the similar looking Chironomidae and Tipulidae are not.

Three subfamilies are recognized within the family Culicidae: Toxorhynchitinae, Anophelinae and Culicinae. Toxorhynchitinae includes only one genus, while Anophelinae consists of nearly 400 species and the subfamily Culicinae contains more than 2,600 mosquito species. More than 40 genera and 3,000 mosquito species are distributed worldwide (table 1) (27), showing a strong capacity to easily adapt to different environmental conditions. Some of these species are more clinically relevant than others, because they affect humans directly, or indirectly. These species include, but are not limited to, *A. aegypti*, *A. vexans* and *Culex quinquefasciatus* (28-30). Adult female mosquitoes require blood-feeding to produce eggs. In this process, they bite and inject saliva before sucking the victim's blood (31). Mosquito saliva contains various substances such as lysozymes, antibacterial glucosidases, anticoagulants, antiplatelet aggregating factors and vasodilators (32-36). It also contains allergens that may induce local cutaneous reactions and, in some cases, a systemic response in atopic individuals (37-40). Allergens derived from the emanations and detritus of mosquitoes are also present in the air and in house dust, and may be inhaled and induce various immune responses (41, 42).

Table 1. Taxonomical classification and distribution of the main mosquito species associated with mosquito allergy.

Subfamily Tribe	Genera	No. of sub-genera	No. of species	Distribution	Species associated with mosquito allergy
Anophelinae	*Anopheles*	7	455	Cosmopolitan	*Anopheles (An.) stephensi, An. minimus, An. sinensis*
	Bironella	3	8	Australasian	
	Chagasia	-	4	Neotropical	
Culicinae					
Aedeomyiini	*Aedeomyia*	2	6	Afrotropical, Australasian, Oriental, Neotropical	
Aedini	*Aedes*	23	363	Old world, Nearctic	*Aedes (Ae.) aegypti, Ae. vexans, Ae. communis, Ae. togoi, Ae. albopictus, Ae. triseriatus*
	Argimeres	2	58	Australasian, Oriental	
	Ayurakitia	-	2	Oriental	
	Borichinda	-	1	Oriental	
	Eretmapodites	-	48	Afrotropical	
	Haemagogus	2	28	Principally neotropical	
	Heizmannia	2	39	Oriental	
	Ochlerotatus	22	550	Cosmopolitan	*Ochlerotatus (Oc.) triseriatus, Oc. hendersoni*
	Opifex	-	1	New Zealand	
	Psorophora	3	48	New world	
	Udaya	-	3	Oriental	
	Verrallina	3	95	Principally Australasian, Oriental	
	Zeugnomyia	-	4	Oriental	
Culicini	*Culex*	23	763	Cosmopolitan	*Culex (Cx.) quinquefasciatus, Cx. tritaeniorhynchus, Cx. pipiens, Cx. pipiens pallens, Cx. tarsalis*
	Deinocerites	-	18	Principally neotropical	
	Galindomyia	-	1	Neotropical	
	Lutzia	3	7	Afrotropical, Australasian, Oriental, Neotropical, Palearctic oriental	
Culisetini	*Culiseta*	7	37	New world, Nearctic	*Culiseta inornata*
Ficalbiini	*Ficalbia*	-	8	Afrotropical, Oriental	
	Mimomyia	3	44	Afrotropical, Australasian, Oriental	
Hodgesiini	*Hodgesia*	-	11	Afrotropical,	

				Australasian, Oriental
Mansoniini	*Coquillettidia*	3	57	Old world, Neotropical
	Mansonia	2	23	Old world, Neotropical
Orthopodomyiini	*Orthopodomyia*	-	38	Afrotropical, Nearctic, Neotropical, Oriental, Palearctic
Sabethini	*Isostomyia*	-	4	Neotropical
	Johnbelkinia	-	3	Neotropical
	Kimia	-	5	Oriental
	Limatus	-	8	Neotropical
	Malaya	-	12	Afrotropical, Australasian, Oriental
	Maorigoeldia	-	1	New Zealand
	Onirion	-	7	Neotropical
	Runchomyia	2	7	Neotropical
	Sabethes	5	38	Neotropical
	Shannoniana	-	3	Neotropical
	Topomyia	2	54	Principally Oriental
	Trichoprosopon	-	13	Neotropical
	Tripteroides	5	122	Principally Australasian, Oriental
	Wyeomyia	15	140	Principally neotropical
Toxorhynchitini	*Toxorhynchites*	4	88	Afrotropical, Australasian, Neotropical, Palearctic oriental, Oriental
Uranotaeniini	*Uranotaenia*	2	265	Afrotropical, Australasian, Oriental, Neotropical

C. The mosquito species *Aedes aegypti*

There are more than 3,000 mosquito species, but some of them represent a problem of higher relevance for humans for their ability to invade areas with different environmental conditions and their capacity to act as vectors for pathogens that cause diseases. *A. aegypti* is one of those species. *A. aegypti* is found in North, Central and South America, Africa, Asia and Oceania (43) and is very abundant throughout tropical and subtropical regions of America, Africa, and Asia, as well as in the Indian Ocean islands, and northern Australia (44) (Figure 4). Although it is not recognized that *A. aegypti* has invaded Europe, there are some studies that have shown its transient presence in some European countries (45-47).

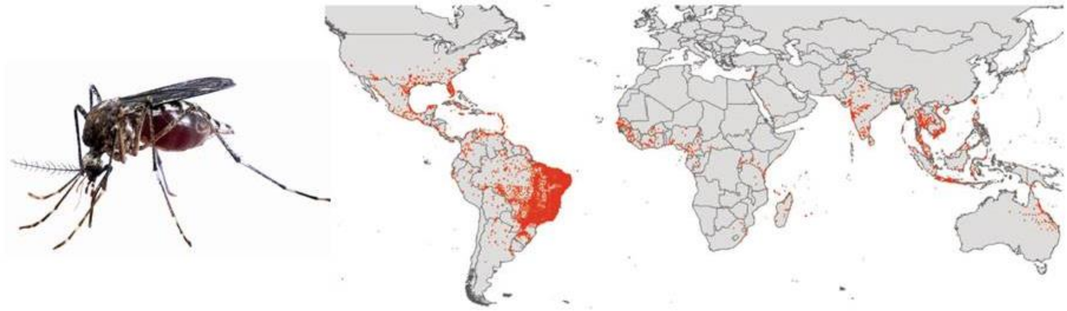

Figure 4. Global distribution of *A. aegypti*. Modified from (43).

A. aegypti is particularly specialized in feeding on humans. It originated in the forest areas of sub-Saharan Africa as a “wild”, black-pigmented insect biting species known as *A. aegypti formosus*. This species is still found in the forest of this region. As a cause of the global spread of *A. aegypti*, facilitated by the commercial trades and the growing capacity of humans to move to different countries, a brown-pigmented species, namely *A. aegypti aegypti,* evolved to specialize in biting and feeding on humans (28, 48). This species has been reintroduced along the coast of East Africa and is considered a ‘domestic’ species (49). In the Rabai region of Kenya, domestic *A. aegypti* readily enter homes, prefer to lay eggs in nutrient-poor river and rain water stored in containers indoors (50). They are resistant to starvation as larvae (51) and have developed a strong preference for biting humans (50, 52). Black-pigmented populations of the native African subspecies *A. aegypti formosus*, known in Rabai as the ‘forest’ species, were found just hundreds of meters away, avoiding homes, laying their eggs in tree holes and rock pools outdoors, and preferring to bite non-human animals.

McBride et al., confirmed the preference of domestic *A. aegypti* for humans. They demonstrated that this behavior is related to the over-expression of Odorant Receptors (but not ionotropic receptors, or Odorant binding proteins) in the antennae of the insect, codified by the gene *Or4*, that recognizes the human volatile odorant Sulcatone (53). The evidence clearly shows that humans are at high risk of being exposed to females of *A. aegypti*, specifically to the saliva antigens released during mosquito bites. The presence of this insect in human homes suggests that other non-saliva-derived antigens may be present in the environment and that humans can be exposed to them through other routes of exposure. In this case, not only the female *A. aegypti* mosquito might constitute a source of antigens, but also other male mosquito species.

D. Allergic reactions induced by mosquito bites

When an atopic individual is bitten by a mosquito, a “normal” inflammatory local reaction consisting of a rash and heat emanation, with the subsequent appearance of a wheal and flare, and itching, appears. However, in atopic individuals, mosquito bites induce severe local, or systemic atypical reactions (ecchymotic or vesiculated), such as anaphylaxis, angioedema, generalized urticaria or wheezing (54, 55). Local cutaneous reactions consist of immediate wheals and flares peaking at 20 min and delayed pruritic indurated papules peaking at 24–36 h (56, 57). Large local reactions consist of itchy red, warm swellings appearing within minutes. Itchy papules and ecchymotic, vesiculated, blistering, bullous or even Arthus-type reactions may appear from 2 to 6 h after the bites (58, 59). Mosquito bite induced allergic reactions are classified in four types: (1) local reactions (immediate and delayed), (2) severe, delayed reactions with scar formation (via a cell-mediated mechanism), (3) exaggerated local reactions, and (4) systemic anaphylaxis (60).

Papular urticaria may also appear as a hypersensitivity reaction after mosquito bites, or after the bites of other arthropods. Some evidence supports the involvement of type I, III and IV hypersensitivity reactions and, therefore, the participation of IgE and IgG antibodies (61, 62). Immunoglobulin and complement depositions have also been detected in the skin (63, 64). In some cases, the mosquito bite may also induce a strong reaction involving an extensive part of the body such as the face, hands, arms, feet, or legs. These reactions appear a few hours after a mosquito bite, which makes it unlikely to be due to a bacterial infection. Simons and Peng coined this clinical disorder as the Skeeter syndrome (54).

The allergic immune reaction raised against saliva-derived mosquito allergens follows a mechanism that occurs after the first exposure, followed by the induction of the typical signs and symptoms of an allergic reaction after subsequent bites. When the affected individuals are chronically exposed, they experience desensitization. The natural history of sensitization and desensitization to mosquito bites has been classified in 5 well-defined clinical stages: (1) no reaction after the initial bite in individuals who have never been bitten, (2) delayed cutaneous lesions appear after approximately 24 h in individuals after subsequent bites, (3) wheals appearing within approximately 15 min in individuals after repeated bites, (4) no delayed reactions occur and only immediate wheals are noted in individuals after further exposures to mosquito bites, and (5) there is a loss of the immediate reaction in individuals who are repeatedly exposed to a large number of mosquito bites (65-67).

The immune response raised against saliva-derived mosquito allergens involves humoral and cellular components (Figure 2). When a non-allergic individual was exposed to 100 bites of *C. quinquefasciatus* every 2 weeks for 10 months, the levels of specific IgE and IgG antibodies increased with each exposure and developed a positive skin prick test (SPT) against a mosquito extract, with a wheal size which correlated with serum antibody levels (56). The reaction disappears after 21 weeks of exposure and the antibody levels decreased (68).

In real life, the process of sensitization and desensitization requires more time because the individuals try to avoid contact with mosquitoes. Kulthanan et al. (55) showed that the required interval between sensitization and desensitization is of approximately 4 years in a population in Thailand. The participation of specific IgE in the local, systemic, immediate and delayed reactions has been demonstrated (38, 69-71). The role of IgG seems to be restricted to the immediate local reactions, but not to the systemic reactions (72). In the early allergic response, in addition to the antibodies the levels of histamine and leukotriene C4 also increased (73).

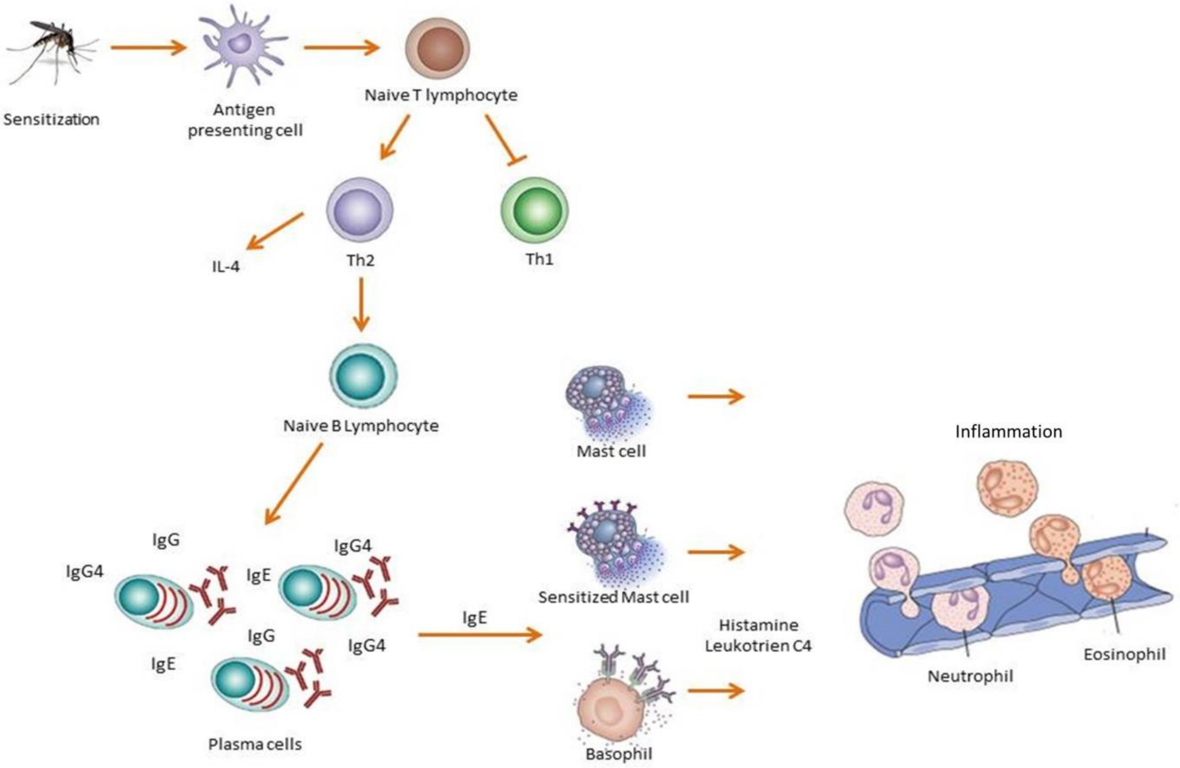

Figure 2. Immune allergic response induced by mosquito bites. Image prepared by JFC.

The immune response of saliva-derived allergens has been studied in animal models of mosquito allergy, showing controversial results. A study showed that *A. aegypti* bites induce a Th2 immune response with immediate and late reactions, higher levels of IgE and IgG1 and lymphoproliferation (74). The allergic reaction may also induce mast cell degranulation. This induces changes in vascular permeability, the extravasation of fluid and the recruitment of neutrophils. This response is accompanied by hyperplasia of the lymph nodes and the recruitment of dendritic cells, B lymphocytes, macrophages and T lymphocytes (75). T cells seem to play a role in the late responses induced by *A. aegypti* bites, since passive transfer of spleen cells, but not serum from mice sensitized with salivary components of *A. aegypti*, induced these reactions (76).

The immune response induced by mosquito bites in animal models varies according to the degree of exposure. In an animal model of allergy caused by *A. aegypti* saliva allergens, mice were exposed four times to approximately 50 female mosquitoes at 14-day intervals, to resemble the natural mechanism of cutaneous sensitization (77). Mosquito sensitized mice produced IL-4, IL-5 and IL-13, but not IFN-γ or IL-17 in bronchial alveolar lavage fluids and showed signals of airway remodeling. In contrast, the mice didn’t experience smooth muscle hyper-reactivity. In addition, high titers of IgE, IgG1 and IgG2a were also observed. In another study, Chen et al. (74) and Wang et al. (78), showed that mice exposed to 6 female mosquito bites, twice a week for four weeks, induced IgE and IgG1 but not IgG2a production. These results suggest that low intensity/acute exposure could preferentially induce antibodies of the Th2 profile, while an intense/chronic exposure is able to induce a mixed pattern with both Th1/Th2 antibody productions.

These results correlate with the natural history of sensitization/desensitization. In the mosquito sensitization process, humans present an early wheal reaction, which disappears following the natural course of the immune response. The mechanisms involved in the desensitization process to mosquito allergens are still unknown. However, it may be partially explained by the induction and presence of specific IgG during the immune response, which may play a protective role.

E. Cross-reactivity

The classic concept of cross-reactivity indicates that this phenomenon occurs when the antibodies induced by exposure to an antigen react with a different protein with structural homology to the antibody-inducing molecule. In the case of allergens, these molecules belong to highly conserved protein families (as is the case for tropomyosin and profilins), which are present in different species and share cross-reactive IgE epitopes. These allergens are named pan-allergens, and are responsible of co-sensitization to different species. Allergenic cross-reactive responses may have an important clinical relevance. For example, patients sensitized to shrimps are very likely to have a positive skin prick test response to other crustaceans (79). Patients with oral allergy syndrome may become sensitized to pollen proteins through inhalation and then experience an IgE-mediated allergic reaction against food antigens that are similar in structure to the pollen proteins (80, 81). In the context of immunotherapy, cross-reactivity may be advantageously exploited by the treatment with a single molecule, or a single allergenic species which may induce desensitization and protection against other similar species. Nowadays, cross-reactivity represents a major diagnostic and therapeutic challenge.

Cross-reactivity has been well documented at the antibody level. However, it can also involve T-cell epitopes with other possible clinical implications. Westernberg et al*.* (82) reported that epitope conservation across allergen species is the major determinant of immunogenicity and the driver of poly-sensitization in allergic patients. They showed that within and beyond the dominant IgE binding allergens, there are peptides and antigens that are highly preserved across pollens and that this conservation is positively correlated with their likelihood to elicit an immune response. On the basis of these observations, they hypothesized that cross-reactive T cells elicited by allergen exposure will: 1) be boosted and selectively expanded by exposure to additional allergens containing the preserved epitope and, 2) generate help for any B cell specific for an allergen cross-reactive at the T-cell level through a classic antigen bridge–linked T-cell/B-cell help mechanism, regardless of whether the IgE response is cross-reactive. Two structurally related molecules may share cross-reactive B epitopes, inducing antibody-mediated immunological responses. However, the exposure to the cross-reactive homologue allergen doesn’t imply that clonal expansion occurs if cross-reactive T-epitopes are missing.

Cross-reactivity involving mosquito-derived allergens has been scarcely studied and mainly focused on salivary antigens. The sera from individuals allergic to mosquito bites react with several common proteins present in the saliva, salivary gland and whole body extracts of *C. quinquefasciatus, A. aegypti, A. albopictus* and *Anopheles minimus* (42). This high degree of cross-reactivity has resulted in apparent sensitization to some mosquito species to which the affected individuals had not been previously in contact (70, 83). The D7 protein family related allergen, Aed a 2, from *A. aegypti*, is one of the most important cross-reactive allergens related to mosquito bite allergy, which is involved in more than 70% of the cases (84). Cross-reactivity between salivary gland extracts of *C. quinquefasciatus, O. triseriatus* and several species of *Aedes* has also been demonstrated in immunized rabbits, where the allergens Aed a 1 and Aed a 2 play an important role (38, 70).

Some individuals may also develop the so-called 'wasp/mosquito syndrome'. Individuals afflicted with this syndrome have specific IgE against a 44-kDa hyaluronidase allergen in mosquitoes and wasps (85). The existence of cross-reactivity between chironomids and mosquitoes has also been suggested in individuals allergic to chironomids (86). A study conducted in Reykjavik, Iceland, showed that most individuals sensitized to *D. pteronyssinus* also had specific IgE against mites (*Lepidoglyphus destructor*), shrimps, cockroaches and mosquitoes (87).

F. Role of tropomyosin in allergic cross-reactivity

Tropomyosins belong to a two-stranded alpha-helical coiled-coil protein family (Pfam PF00261) found in cell cytoskeletons. They are integral components of actin filaments that play a critical role in regulating the function of actin filaments in muscle and non-muscle cells. Muscle tropomyosin isoforms are involved in regulating the interactions between actin and myosin. Non-muscle tropomyosin isoforms function in all cells and are involved in a range of cellular pathways that control and regulate the cytoskeleton and other key cellular functions. These proteins consist of rod-shaped coiled-coil hetero-, or homo-dimers that lie along the α-helical groove of most actin filaments. There is no three-dimensional structure available from any allergenic tropomyosin, but a predicted model of *A. aegypti* tropomyosin (Uniprot: Q17H75) is presented in Figure 3.

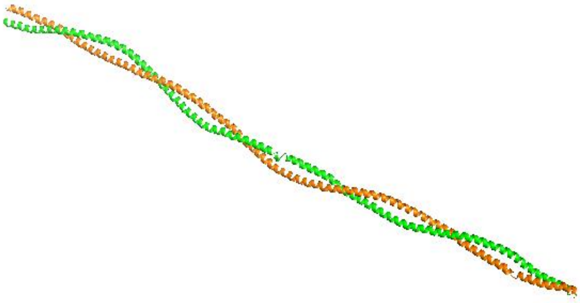

Figure 3. Molecular model of *A. aegypti* tropomyosin generated by Swiss-Model (template PDB 1c1.2.B).

Tropomyosin is a well described allergen from diverse sources. There are several tropomyosin allergens currently registered in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature data set (http://www.allergen.org) and Allergome database (http://www.allergome.com/). Most of the allergenic sources are shrimps, lobsters, prawns, crabs, fish, Mollusks and snails. This allergen is also common in mites, helminths, cockroaches and other insects.

A phylogenetic analysis conducted by Leung et al. (88) found that tropomyosins in arthropods share a 91.7% homology (76.1–100%) and in mollusks 77.2% (65.1–99.3%). Homology between crustacean and mollusks tropomyosins ranges from 56 to 68% and between vertebrate and invertebrate from 53 to 57% (89). This high degree of homology indicates that tropomyosin is a pan-allergen, as has been confirmed and described in several studies (79). Although tropomyosin is considered an important allergen, only a few studies have demonstrated its role in the allergic response. Sensitization can occur by ingestion (seafood), inhalation (mites, cockroaches), or parasite infection (Ascariasis, Anisakiasis). The clinical impact of *Ascaris lumbricoides* tropomyosin (Asc l 3) was demonstrated in a population from a tropical country (90, 91).

The IgE frequency of sensitization to tropomyosin is variable, but usually low. Tropomyosin from shrimp, Pen a 1, binds up to 75% of all shrimp-specific IgE antibodies (89, 92). In Africa and South America, the prevalence of sensitization to mite tropomyosin is approximately 50% (93, 94), higher than in developed countries (95, 96).

There are studies directed to identify B and T epitopes from allergenic tropomyosins. Ayuso et al. (97, 98) identified several IgE binding sites using peptides covering the whole sequence of the shrimp tropomyosin Pen a 1. Eight of these peptides corresponded to 5 sites that contained cross-reactive epitopes in shrimp, lobster, house dust mite, and cockroach. Epitope 1 (residues 43–55) in region 1; epitope 2 (residues 87–101) in region 2; epitopes 3a (residues 137–141) and 3b (residues 144–151) in region 3; epitope 4 (residues 187–197) in region 4; and epitopes 5a (residues 249–259), 5b (residues 266–273), and 5c (residues 273–281) in region 5. These regions had from 56% to 98% of homology within other tropomyosins. Ravkov et al. (99) reported 17 T-cell epitopes from shrimp tropomyosin restricted to multiple MHC class II alleles, which is an important data for the design of peptide-based immunotherapy of shrimp-allergic patients.

The genome of *A. aegypti* contains 11 genes that codify for different variants, or isoforms of tropomyosin (100). The amino acid sequences of 11 tropomyosin proteins deduced from these genes are deposited in the protein database Uniprot. Currently, there are no studies reporting the identification, or biochemical and immunological characterization of the native forms of these tropomyosins.

G. Reported *A. aegypti* allergens

Atopic individuals exposed to mosquito develop an allergic response to different allergens contained in the saliva, salivary glands and the insect body. Four saliva-derived allergens from *A. aegypti* have been reported in the WHO/IUIS Allergen database (Table 2).

Table 2. Reported *A. aegypti* allergens

Allergen	Molecular weight (kDa)	Biological function	Number of aminoacids	Frequence of reactivity (% positives)	
				IgE	Skin prick test
Salivary allergens previously reported					
Aed a 1	68	Apyrase	562	--------	29 - 43
Aed a 2	37	Salivary D7 protein	321	43	11
Aed a 3	30	Undefined 30 kDa salivary protein	273	--------	32
Aed a 4	67	α-glucosidase	579	36	--------
Novel non-salivary allergens reported herein					
Aed a 5	17.9	Sarcoplasmic Ca+ (EF-hand) binding protein	191	26.2	--------
Aed a 6	24.7	Porin 3	282	33.3	--------
Aed a 7	27.4	Undefined protein	204	26.6	--------
Aed a 8	69	Heat Shock cognate protein-70	655	60	--------
Aed a 10	32	Tropomyosin	285/284*	33.3	--------
Aed a 11	32.6	Lysosomal aspartic protease	387	40	--------

*Two variants (Aed a 10.0101 and Aed a 10.0201) are reported for tropomyosin

1. Saliva-derived *A. aegypti* allergens

a. Aed a 1

This 68 kDa allergen is a member of the apyrase (ATP diphosphohydrolase) protein family, expressed exclusively in the mosquito saliva. It interferes with platelet aggregation in human blood, hydrolyzing ADP and ATP released by platelets and other cells (101). It shows homology with members of a family of enzymes with 5′-nucleotidase activity (102). The capacity of this molecule to block platelet aggregation has further encouraged its study for the treatment of thrombosis (32). In one study, 9 of 31 allergic patients (29%) sensitized to *A. aegypti* had a positive skin test to rAed a 1 (39). IgE and IgG reactivity testing suggested that C-terminal residues (residues 150–562) from this allergen contain B cell epitopes (103). Its biological activity was demonstrated in immunized rabbits which produced antibodies that recognized the native molecule in *A. vexans* and *A. albopictus* (70).

b. Aed a 2

This allergen belongs to the D7 protein family, which are highly conserved in insects that require blood feeding for reproduction. As it is the case with Aed a 1, this allergen is exclusively expressed in the salivary glands of female mosquitoes (104). Aed a 2 contains a N-terminal and a C-terminal domain, which bind to leukotrienes and biogenic amines that are released as a mechanism of defense against the mosquito bites (105). The gene encoding this protein was first identified in a cDNA library of *A. aegypti* salivary glands and obtained as a recombinant protein by expression in insect cells infected with baculovirus, which conserved antibody binding capacity and skin reactivity as the natural protein. Immunogenic and allergenic properties of rAed a 2 have been demonstrated in mice, which produced specific IgG1 and IgE that reacted with the recombinant and natural allergens. Sensitized mice showed a positive skin test to the recombinant allergen (106). The presence of homologues of Aed a 2 and antibody cross-reactivity assays showed that this allergen has allergenic cross-reactivity with proteins from other mosquito species (70, 107).

c. Aed a 3

This allergen has been obtained by molecular cloning and expression in insect cells infected with baculovirus. Aed 3 is expressed as a 30-kDa protein which binds specifically to collagen and prevents the interaction of collagen with platelet glycoprotein IV, integrin α2β1 and von Willebrand factor (108), making it a potential molecule with antithrombotic capacity. It has been suggested that this allergen may also be used for the diagnosis of mosquito allergy.

d. Aed a 4

This 67 kDa allergen is an α-glucosidase which has been obtained as a recombinant protein by expression in insect cells infected with baculovirus and showed IgE and IgG binding capacity; 46% of 13 allergic individuals had a positive specific IgE determination against Aed a 4; healthy individuals showed no IgE reactivity (109).

rAed a 1, rAed a 2 and rAed a 3 are among the most relevant allergens, that can be used for the diagnosis of mosquito bite allergy. A mixture of these three allergens is enough to identify approximately 65% of mosquito-allergic patients (110). The clinical relevance of other allergens remains to be studied.

2. Body-derived *A. aegypti* allergens

As a result of this project, 6 novel *A. aegypti* allergens where included in the WHO/IUIS Allergen database: Aed a 5 (Sarcoplasmic Ca+ (EF-hand) binding protein), Aed a 6 (Porin 3), Aed a 7 (Undefined protein), Aed a 8 (Heat Shock cognate protein-70), Aed 11 (Lysosomal aspartic protease) and two variants of tropomyosin (Aed a 10.0101 and Aed a 10.0201).

H. Other diseases induced or transmitted by mosquito bites

Mosquito bite allergy is associated with other diseases. Children and individuals with primary or secondary immunodeficiency are most at risk for mosquito-bite allergy (59, 111, 112). Several cases have been described in which sensitization to mosquito bites is accompanied with natural killer (NK) cell lymphoproliferative disorders, the presence of T cells infected with Epstein-Barr virus, or lymphocytosis associated with HIV infection (113-116). The study by Asada et al. (117) demonstrated that mosquito saliva induces lymphoproliferation, increasing the expression of an oncogene in NK cells, which apparently plays an important role in the etiology of mosquito allergy.

It has been shown that the inflammatory and/or allergic response induced by mosquito bites, especially *Anopheles* spp., favors infection with *Plasmodium* spp., thereby facilitating the occurrence of malaria (118, 119). Therefore, mosquito-sensitized individuals seem to be at a greater risk of contracting malaria. *A. aegypti* is responsible for transmitting many medically important viruses, such as those which cause Zika, Chikungunya and dengue. *A. aegypti* is the main cause of dengue problems in Central and South America, Southeast Asia, and the Western Pacific, with an estimated 50 million infections every year (120).

The role of the mosquito is not only as a virus carrier. Using Semliki Forest virus and Bunyamwera virus, Pingen *et al* (121) showed that these viruses exploit the inflammatory reaction induced after the mosquito bite and the innate immune response driven by neutrophils to replicate and disseminate *in vivo,* and infect the virus-permissive myeloid cells.

I. Can mosquitoes induce allergic reactions by non-saliva derived antigens?

Although there is no clear evidence about this possibility, there are several reasons to believe that mosquitoes have body-derived allergens that may induce allergic responses, other than cutaneous eruptions. First, allergic individuals suffering from asthma and/or allergic rhinitis have specific IgE against mosquito antigens contained in whole body extracts. In a study conducted in India, it was shown that 47% of the population with asthma and/or allergic rhinitis were sensitized to mosquito allergens, as determined by skin-prick tests, serum IgE antibodies and bronchial provocation tests with whole mosquito body extracts (122). A study performed with Indian patients suffering from bronchial asthma and allergic rhinitis showed that the diagnosis of these allergic responses with mosquito extracts is reliable (123). Second, mosquito body particles (detritus) may remain suspended in the ambient air. In these circumstances, individuals may inhale mosquito debris and develop an allergic response (41, 124). Third, clinical symptoms have been reported upon the inhalation of other insects, especially in occupational and urban settings. The mechanism associated with the inhalation of mosquito allergens may be similar to those implicated with other insects that induce, or exacerbate, allergic asthma and rhino-conjunctivitis upon inhalation of their allergens. Airborne allergens from cockroaches (125-127), moths (128) and honeybees (129) have been detected in settled dust and air of homes, schools and working places in the USA. The presence of these allergens is associated with the development of allergic responses. Cases of allergic sensitization to the common housefly *Musca domestica,* where a cross-reactive component was discarded, have been reported (130). Fourth, the analysis of the *A. aegypti* genome indicates that this insect expresses several proteins homologues of allergens from mites, cockroaches, or shrimps, which may play a role in the pathophysiology of the allergic responses by cross-reactivity, or specific sensitization. This is a clear case for molecules such as tropomyosin (with homologues in several arthropods), heat shock protein-70 (with homologues in mites, mold, yeast and wasp) and peptidyl-prolyl isomerase (with homologues in mites, mold, plants and yeast). Figure 5 shows the distribution of allergenic arthropods in indoor dust.

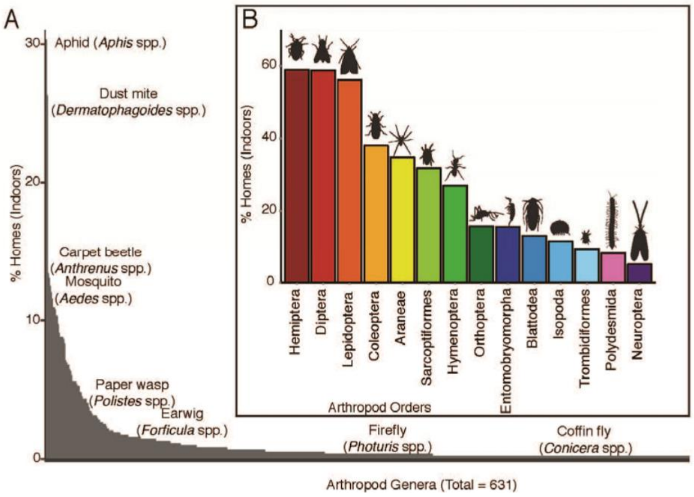

Figure 5. Distribution of select allergenic arthropods in indoor dust. Modified from (124).

The study of Kausar et al. (41) showed important contributions to the raised explanations and identified two allergens of 30 and 33 kDa which may correspond to major mosquito body allergens. The evaluation of specific IgE-sensitization to a whole body extract from the mosquito species *C. quinquefasciatus* in 200 allergic patients with bronchial asthma and/or allergic rhinitis showed that 35% had positive a skin prick test and a positive specific IgE determination. RAST inhibition experiments with *Spodoptera* spp., *Prosopis* spp., *Ricinus* spp., *Alternaria* spp., *Mucor* spp., horse dander, and *M. domestica* extracts did not inhibit the *C. quinquefasciatus* extract, suggesting that this sensitization was not produced by cross-reactivity with these species. However, whole body extracts of *A. aegypti* and *A. stephensi* produced a similar inhibition comparable to *C. quinquefasciatus*. IgE reactivity against *C. quinquefasciatus* using an extract prepared from airborne particles showed that mosquito antigens remain suspended in the air. Another study (124) reported that mosquitos (*Aedes* spp.) and plant-associated aphids (*Aphis* spp.) were two of the most ubiquitous Arthropods detected in the dust from homes in the United States (Figure 5).

J. The study of allergens in the "omic era"

Classic approaches to the identification and characterization of allergens include the purification from natural sources (131-134) and the construction of cDNA libraries from which the nucleotide sequence codifying the allergen of interest can be detected, manipulated and used to express the recombinant protein (135-137). In this project, we have identified and characterized a novel *A. aegypti* allergen (Aed a 10: tropomyosin) by the purification of the natural protein, which has advantages over other techniques, as long as the availability of the source of allergen material is not limited. The application of these techniques is time-consuming and may lead to a poor identification of the most relevant spectrum of allergens, which are not detected, or underestimated.

The advent of the "omic era" has opened new perspectives that applied to the study of allergens allows a more comprehensive identification and characterization of the allergens from a species, together with information of their abundance, pos-translational modifications, physicochemical characteristics and interaction in the scene of the biochemical pathways. Furthermore, the development to next-generation sequencing technologies has provided the tools to achieve a massive parallel sequencing, leading to a more rapid and efficacious way to sequence genomes, analyse the expression of genes and identify polymorphisms that may lead to the expression of allergen isoforms with variable allergenic and immunologic characteristics.

A good example which shows the advantages of the omic tools applied to the identification of allergens was conducted with the house dust mite *D. farinae* (138). The presence of 25 of the previously described *D. farinae* allergens registered in the International Union of Immunological Societies Allergen Nomenclature Sub-Committee database was confirmed. Furthermore, 12 novel allergenic proteins, including ferritin, cofilin (an actin-binding protein), secreted inorganic pyro-phosphatase (denominated Der f 30-32, respectively), ubiquinol–cytochrome c reductase binding protein–like protein (denominated Der f 24), myosin alkali light chain protein and DFP2, were identified. The methodological approach combined high-throughput sequencing of the genome and transcriptomic, and the characterization of IgE-binding components of the mite using proteomic tools. In addition to the identified allergens, the microbiome of

D. farinae was also described. This information is valuable for the allergologists because provides the bases to better understand the allergenicity of the mite molecules in the context of the internal and external environment. The results from the analysis of the genome and transcriptome from this mite species helped to propose a list of potential allergens that present allergen homologues in *Dermatophagoides spp.* and other mite species. The results presented by Chan TF et al. shows a long list of allergens from mites; some of them are potential molecular markers of genus or species and others are cross-reactive molecules.

In a similar approach, the allergens from *Tyrophagus putrescentiae* were identified by next-generation sequencing combined with MS/MS shotgun proteomics. This analysis led to the identification of three previously-uncharacterized allergens, Tyr p 28, Tyr p 35, and Tyr p 36, which have been officially assigned by the WHO/IUIS Allergen Nomenclature Sub-committee (139).

Proteomic is defined as the in-depth analysis of protein repertoires within a given species, organ, or organelle, at a given time (140). This definition applied to the study of allergens, has led to the concept of “Allergenome”. The application of proteomics in the field of Allergy research is of great interest for the identification and structural characterization of allergens and the study of immune epitopes. Furthermore, proteomics can provide semi-quantitative or quantitative information about these molecules in different allergen extracts. Application of proteomic tools to the study of allergens is also advantageous because it involves the study of the molecules with their natural post-translational modifications, variants and isoforms (141-143) and gives information of their biological relevance. For instance, in an attempt to find homologues of the birch pollen allergen, Bet v 1 in the kiwi fruit, the screening of cDNA libraries allowed the identification of the allergens Act d 8 and Act c 8 from two kiwi species (144). However, they were not relevant because of their very low representation in the kiwi extracts. Analysing the protein content of this fruit, it was found that other Bet v 1 homologue, namely the allergen Act d 11, is contained in high concentrations in the allergenic extracts, representing an important and clinically relevant cross-reactive allergen (145).

At the experimental level, the proteomic study of allergens requires the separation of a complex spectrum of proteins. The task may be accomplished using bi-dimensional polyacrylamide gel electrophoresis (2D-PAGE), but other high resolution methods based on electrophoretic or chromatographic separations can also be applied. Afterwards, antibody reactive proteins can be identified by immunological methods and characterized by mass spectrometry. Biological samples are first subjected to isoelectric focusing (i.e., first dimension) and further separated according to molecular masses (i.e., second dimension). Separated proteins are identified with colorimetric reagents (Coomassie blue or silver staining) and incubated with sera collected from allergic individuals in Western blot experiments. Antibody-binding spots are further analyzed by MS/MS, exploiting the information from protein databases, when possible. If no reliable results are obtained using protein databases, transcriptomic information (i.e., data from RNA sequencing) is highly valuable to identify new allergens of clinical relevance. These combined approaches based on “omics” technologies are almost enough to identify every possible allergen, as it is the case with grass pollen (146), ragweed (147) and mites (148). There are two main approaches to analyze proteins by proteomic tools: the bottom-up approaches, where proteins are separated, digested with an enzyme (usually using trypsin), and the resulting peptides identified by MS and the top-down (where complete proteins are analyzed) (149) (Figure 6).

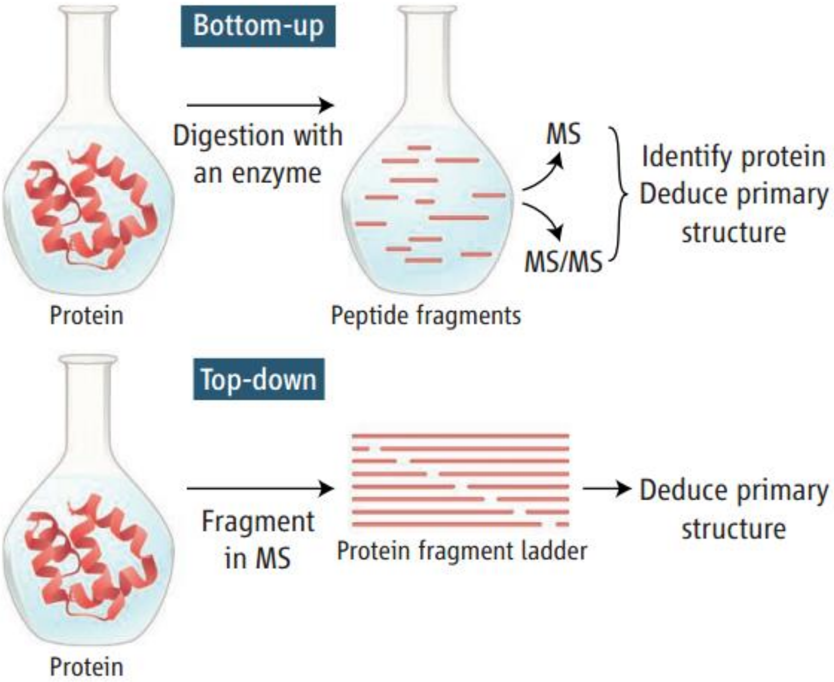

Figure 6. Top-down and bottom- up strategies for mass spectrometry identification of proteins.

Bottom-up strategy, which is the most frequently used, can be performed by two different procedures. Peptide mass fingerprint (PMF) which requires separation of proteins by 2D-PAGE, enzymatic digestion into peptides and MS analysis (149). The obtained peptide mass profile is compared against generated in-silico digestion of the protein sequences available in databases (150). A second approach is the peptide fragmentation fingerprint (PFF), which produces fragmentation data from one, or more unique peptides to unambiguously identify parent proteins (149) and produce more confident results (150).

Choopong et al. (151) studied the proteome and allergenome of the mite species *D. farinae*, using SDS-PAGE (1DE) and 2DE-IgE immunoblotting followed by LC-MS/MS and database search for protein identification. They identified 40 IgE-binding spots corresponding to 35 different proteins. Some of these proteins matched previously reported allergens. Several minor allergens that have never been previously reported were also identified, including deoxyribonuclease-2-beta, alpha-enolase, fructose biphosphate aldolase, glyceraldehydes 3-phosphate dehydrogenase, phosphoenolpyruvate carboxylase, NADH dehydrogenase, cytochrome-b, aldehyde dehydrogenase, or Alt a 10, actins, heat shock protein-60, glutamate dehydrogenase, ATP synthetase, citrate synthase, phosphoglycerate kinase, serine hydroxymethyl transferase, transketolase-like protein, and hexoseaminidase.

The proteome and transcriptome of the mosquito species *A. aegypti* have been previously studied to: a) discover potential receptors for insecticidal proteins (152, 153), b) to understand the mechanisms of infection for some viruses (154, 155), c) to study the molecular mechanisms associated to the insect bite (156, 157) and d) to find novel approaches to control the infestation of this mosquito (158). However, there is no study that uses proteomic tools to contribute to the elucidation of its allergenic components and understanding of the role of *A. aegypti* in allergic diseases.

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Review

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Immunological Aspects of the Immune Response Induced by Mosquito Allergens

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Key Words

Mosquito allergy · *Aedes aegypti* · Immunoglobulin E · Recombinant allergens · Cross-reactivity

Abstract

Allergies caused by mosquito bites may produce local or systemic reactions. The inhalation of mosquito allergens may also cause asthma and/or allergic rhinoconjunctivitis in sensitized individuals. The mechanisms implicated in the development of these immune responses involve IgE antibodies, different subtypes of IgG and proinflammatory cytokines as well as basophils, eosinophils and mast cells. Several allergenic components have been identified in the saliva and bodies of mosquitoes and some of these are present in different mosquito species. The most common species implicated in allergic reactions belong to the genera *Aedes*, *Culex* and *Anopheles*. Several *Aedes aegypti* allergens have been cloned and sequenced. The recombinant molecules show IgE reactivity similar to that of the native allergens, making them good candidates for the diagnosis of mosquito allergies. Allergen-specific immunotherapy with mosquito extracts induces a protective response characterized by a decreased production of IgE antibodies, increased IgG levels, a reduction in the severity of cutaneous and respiratory symptoms and the need for medication. The aims of this review are to summarize the progress made in the characterization of mosquito allergens and discuss the types of immune responses induced by mosquito bites and the inhalation of mosquito allergens in atopic individuals.

Introduction

Mosquitoes are insects belonging to the order Diptera and members of a family of the nematocerid flies Culicidae (from the Latin culex, genitive culicis, meaning 'midge' or 'gnat') [1]. The word 'mosquito' (formed by 'mosca' and the diminutive 'ito') is from the Spanish or Portuguese for 'little fly' [2]. Mosquitoes resemble crane flies (family Tipulidae) and chironomid flies (family Chironomidae). The females of many species of mosquitoes are blood-eating pests and dangerous vectors of disease whereas members of the similar-looking Chironomidae and Tipulidae are not.

At least 14 orders of arthropods, containing over 400 different genera and more than 15,000 species, evolved to feed on blood from warm vertebrate animals [3]. Three subfamilies are recognized within the family Culicidae, namely Toxorhynchitinae, Anophelinae and Culicinae. Toxorhynchitinae includes only one genus, while Anophelinae consists of nearly 400 species. More than 2,600 mosquito species belong to the subfamily Culicinae. More

KARGER 125

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than 40 genera and 3,000 species of mosquitoes are distributed worldwide (table 1) [4]. Some of these species are more clinically relevant than others because they affect humans, directly or indirectly. These species include, but are not limited to, *Aedes aegypti, Aedes vexans* and *Culex quinquefasciatus* [5–7]. Adult female mosquitoes require blood-feeding to produce eggs. In this process, they bite and inject their saliva before sucking their victim's blood [8]. Mosquito saliva contains various substances like lysozymes, antibacterial glucosidases, anticoagulants, antiplatelet aggregating factors and vasodilators [9–13]. It also contains allergens that may induce local cutaneous reactions and, in some cases, a systemic response in atopic individuals [14–17]. Allergens derived from the emanations and detritus of mosquitoes are also present in the air and in house dust, and may be inhaled and induce various immune responses [18, 19].

The term 'mosquito allergy' is used to describe a series of signs and symptoms caused by mosquito bites. Individuals may experience a variable degree of symptoms after a mosquito bite consisting of unusual large local ecchymotic and vesiculated reactions and, in some cases, systemic reactions, such as generalized urticaria, angioedema or wheezing and even anaphylaxis [20–23]. However, it has also been shown that mosquito debris remains suspended in the air and produces an allergic respiratory response similar to that caused by other airborne allergens, such as those from the dust mite and cockroach [18, 24, 25].

Allergies caused by mosquitoes have a negative impact on the affected population, on their quality of life and, in some cases, even put their lives at risk. Although the general opinion is that mosquito allergy may be highly prevalent, there are only a few epidemiological studies supporting this perception. In a cross-sectional study conducted in Mexico City, 82% of the patients who were admitted to the allergy service reported that they were allergic to mosquito bites; however, only 2.5% of them showed positive skin reactions to this insect [26]. In another study, conducted in India, it was shown that 47% of the population with asthma and/or allergic rhinitis were sensitized to mosquito allergens, as determined by skin-prick tests, serum IgE antibodies and bronchial provocation tests with whole mosquito body extracts [25]. There is no epidemiological data about mosquito allergy in South America, where several mosquito species and mosquito-borne diseases are highly prevalent.

Mosquito sensitization may also be associated with other diseases. Children and individuals with primary or secondary immunodeficiency are most at risk for mosquito-bite allergy, especially those living in areas heavily exposed to mosquitoes and their allergens [23, 27, 28]. Several cases have been described in which sensitization to mosquito bites is accompanied with natural killer (NK) cell lymphoproliferative disorders, the presence of T cells infected with Epstein-Barr virus or lymphocytosis associated with HIV infection [29–32]. The study by Asada et al. [33] demonstrated that mosquito saliva induces lymphoproliferation, which, in turn, increases the expression of an oncogene in NK cells, which apparently plays an important role in the etiology of mosquito allergy. It has been shown that the inflammatory and/or allergic response induced by mosquito bites, especially *Anopheles* spp., favors infection with *Plasmodium* spp., thereby facilitating the occurrence of malaria [34, 35]. Therefore, mosquito-sensitized individuals seem to be at a greater risk of contracting malaria.

Allergy to house dust mites is one of the most important risk factors for asthma in tropical regions. The main species involved are *Blomia tropicalis* and *Dermatophagoides pteronyssinus* [36–38]. Mosquitoes are abundant in these latitudes, since the climatic conditions of tropical regions favor the life cycle and proliferation of mosquito species [39–42]; this fact might favor high exposure to their allergens. Mosquitoes, like mites, may also be an important environmental factor that induces or exacerbates allergic diseases. Moreover, studies on mosquitoes, mites and cockroaches indicate that these arthropods have some allergens in common [43–46]. These allergens have not been investigated and identified yet, despite the fact that cross-reactivity between mites and mosquito may play a significant role in the prevalence and clinical manifestations of allergic respiratory and dermatological diseases.

Immunological Basis of the Allergic Reactions Produced by Mosquitoes

Symptoms and Clinical Stages of Reactivity

Mosquito bites produce a local reaction consisting of a rash and heat, with the subsequent appearance of a wheal and flare, and itching in nonatopic individuals. The term 'mosquito allergy' refers to reactions which cause severe or atypical (ecchymotic or vesiculated) local or systemic reactions, such as anaphylaxis, angioedema, generalized urticaria or wheezing. Four types of reactions occur as the consequence of a mosquito bite: (1) local reactions (immediate and delayed), (2) severe, delayed reactions

Table 1. Taxonomical classification and distribution of the main mosquito species associated with mosquito allergy (including those to which sensitization has been demonstrated in vivo/in vitro)

Subfamily	Genera	Subgenera, n	Species, n	Distribution	Species associated with mosquito allergy
Anophelinae	*Anopheles*	7	455	cosmopolitan	*A. stephensi, A. minimus, A. sinensis*
	Bironella	3	8	Australasian	
	Chagasia	–	4	neotropical	
Culicinae					
Aedeomyiini	*Aedeomyia*	2	6	Afrotropical, Australasian, Oriental, neotropical	
Aedini	*Aedes*	23	363	Old World, Nearctic	*A. aegypti, A. vexans, A. communis, A. togoi, A. albopictus, A. triseriatus*
	Argimeres	2	58	Australasian, Oriental	
	Ayurakitia	–	2	Oriental	
	Borichinda	–	1	Oriental	
	Eretmapodites	–	48	Afrotropical	
	Haemagogus	2	28	principally neotropical	
	Heizmannia	2	39	Oriental	
	Ochlerotatus	22	550	cosmopolitan	*O. triseriatus, O. hendersoni*
	Opifex	–	1	New Zealand	
	Psorophora	3	48	New World	
	Udaya	–	3	Oriental	
	Verrallina	3	95	Principally Australasian, Oriental	
	Zeugnomyia	–	4	Oriental	
Culicini	*Culex*	23	763	cosmopolitan	*C. quinquefasciatus, C. tritaeniorhynchus, C. pipiens, C. pipiens pallens, C. tarsalis*
	Deinocerites	–	18	principally neotropical	
	Galindomyia	–	1	neotropical	
	Lutzia	3	7	Afrotropical, Australasian, Oriental, neotropical, Palearctic oriental	
Culisetini	*Culiseta*	7	37	New World, Nearctic	*C. inornata*
Ficalbiini	*Ficalbia*	–	8	Afrotropical, Oriental	
	Mimomyia	3	44	Afrotropical, Australasian, Oriental	
Hodgesiini	*Hodgesia*	–	11	Afrotropical, Australasian, Oriental	
Mansoniini	*Coquillettidia*	3	57	Old World, Neotropical	
	Mansonia	2	23	Old World, Neotropical	
Orthopodomyiini	*Orthopodomyia*	–	38	Afrotropical, nearctic, neotropical, Oriental, palearctic	
Sabethini	*Isostomyia*	–	4	neotropical	
	Johnbelkinia	–	3	neotropical	
	Kimia	–	5	Oriental	
	Limatus	–	8	neotropical	

Table 1. (continued)

Subfamily	Genera	Subgenera, n	Species, n	Distribution	Species associated with mosquito allergy
	Malaya	–	12	Afrotropical, Australasian, Oriental	
	Maorigoeldia	–	1	New Zealand	
	Onirion	–	7	neotropical	
	Runchomyia	2	7	neotropical	
	Sabethes	5	38	neotropical	
	Shannoniana	–	3	neotropical	
	Topomyia	2	54	principally Oriental	
	Trichoprosopon	–	13	neotropical	
	Tripteroides	5	122	principally Australasian, Oriental	
	Wyeomyia	15	140	Principally neotropical	
Toxorhynchitini	*Toxorhynchites*	4	88	Afrotropical, Australasian, neotropical, palearctic Oriental, Oriental	
Uranotaeniini	*Uranotaenia*	2	265	Afrotropical, Australasian, Oriental, neotropical	

This table has been modified from data in Harbach [4].

with scar formation (via a cell-mediated mechanism), (3) exaggerated local reactions, and (4) systemic anaphylaxis [47]. Local cutaneous reactions consist of immediate wheals and flares peaking at 20 min and delayed pruritic indurated papules peaking at 24–36 h [48, 49]. Large local reactions consist of itchy red, warm swellings appearing with minutes, itchy papules and ecchymotic, vesiculated, blistering, bullous or even Arthus-type reactions, appearing 2–6 h after the bites [20, 23, 50].

Sensitization may occur after the first exposure to a mosquito bite. Subsequent bites may induce the characteristic signs and symptoms of an allergic reaction. Based on clinical observations, the natural history of sensitization and desensitization to mosquito bites has been classified into 5 well-defined clinical stages: (1) no reaction after the initial bite in individuals who have never been bitten, (2) delayed cutaneous lesions appear after approximately 24 h in individuals after subsequent bites, (3) wheals appear within approximately 15 min in individuals after repeated bites, (4) no delayed reactions occur and only immediate wheals are noted in individuals after further exposures to mosquito bites, and (5) there is a loss of the immediate reaction in individuals who are repeatedly exposed to huge numbers of mosquito bites [51–53].

Evidence suggests that mosquitoes might also induce allergic respiratory symptoms through the inhalation of suspended allergens that derive from their bodies and emanations. The available evidence and similar findings with regard to other flying insects suggest that asthma and allergic rhinitis might be exacerbated by the inhalation of mosquito allergens, in a similar way to other airborne allergens derived from arthropods such as mites, moths [54], bees [55] and cockroaches [56].

Humoral and Cellular Response to Mosquito Bites

Immunological changes that occur during the process of sensitization and desensitization have been studied in humans and in animal models, and involve the humoral as well as the cellular components of the immune response. In a prospective study [48] on the natural course of the allergic response against mosquito bites, a nonallergic individual was exposed to 100 bites of *C. quinquefasciatus* every 2 weeks for 10 months. The levels of specific IgE and IgG antibodies increased steadily with each exposure. Skin tests with a mosquito extract were positive, and the size of the wheal correlated with IgE and IgG antibody levels. However, after 21 weeks of exposure, the levels of specific antibodies to the extract decreased. Although these results demonstrate that an individual may be desensitized to mosquito bites, in real life, such individuals try to avoid contact and thus reduce the number of bites, thereby requiring more time for desensitization. In a population of children from Winnipeg, Man., Canada, high levels of specific IgE and IgG occurred in the first 6 months, but had decreased by adolescence, suggesting that these children were desensitized naturally by exposure [57]. Kulthanan et al. [21] showed the required interval between sensitization and desensitization in a population in Thailand to be approximately 4 years.

The participation of specific IgE in mosquito allergy has been demonstrated by immediate skin reactivity induced by the passive transfer of specific IgE antibodies [58]. These antibodies were associated with local and systemic, and immediate or delayed reactions. It has been demonstrated that specific IgE levels are higher in the serum of individuals allergic to at least 1 of 10 mosquito species distributed worldwide than those in nonallergic individuals [44]. Brummer-Korvenkontio et al. [22] showed that individuals sensitized to *Aedes communis* with immediate and delayed reactivity had high levels of specific IgE and IgG4 to *A. communis*. Peng et al. [14] showed that individuals sensitized to mosquito bites who experienced acute systemic reactions had higher specific IgE levels than nonallergic individuals. In these cases, there was also a positive and significant correlation between the size of the sting wheals and the levels of serum antibodies. It seems that IgG antibodies may play a role in immediate local reactions, but not in systemic reactions. A study by Reunala et al. [59] demonstrated that between 82–90% of children in Finland, Kenya and Mexico had specific IgE antibodies against *A. communis* and *A. aegypti*, with the presence of IgG4 antibodies in the children being 85, 41 and 20%, respectively. The titers of specific antibodies were associated with the degree of exposure. It was demonstrated that in the early allergic response caused by mosquito bites, in addition to the levels of the antibodies, those of histamine and leukotriene C4 also increased [60]. In a mouse model of mosquito allergy, *A. aegypti* induced a Th2 immune response with immediate and late reactions and higher levels of IgE and IgG1 antibodies compared to mice that were nonsensitized. A higher lymphoproliferation was also observed in sensitized mice treated with mosquito extracts [61].

Mast cells may also play a role in allergic symptoms caused by mosquito bites. Demeure et al. [62] showed that the bites of *Anopheles stephensi* activated mast cell degranulation in healthy mice; this induced changes in vascular permeability, the extravasation of fluid and the recruitment of neutrophils at the bite site. This response was accompanied by hyperplasia of the lymph nodes and the recruitment of dendritic cells, B lymphocytes, macrophages and T lymphocytes. These effects were not observed in mast cell-deficient animals, unless they were reconstituted with mast cells. An important finding in this study was that the in vitro degranulation of mast cells occurs in the absence of IgE, suggesting that the saliva may be responsible for the inflammatory response.

Other Manifestations of Mosquito Allergy

Papular urticaria may also appear as a hypersensitivity reaction after mosquito bites, or after the bites of other arthropods. The exact immune mechanisms behind papular urticaria are unknown, but some evidence support the involvement of type I, III and IV hypersensitivity reactions, and therefore the participation of IgE and IgG antibodies. Abdel-Naser et al. [63] showed that a group of patients with papular urticaria had specific IgG against *Culex pipiens* antigens. The levels of these antibodies correlated with the size of both immediate and delayed skin reactions and specific IgE levels [64]. Immunoglobulin and complement depositions have also been detected in the skin, suggesting that these participate in the mechanism of papular urticaria lesions [65, 66].

In some cases, the mosquito bite may also induce a strong reaction involving an extensive part of the body such as the face, hands, arms, feet or legs. These reactions are often misdiagnosed as cellulitis. However, they appear a few hours after the mosquito bites, which makes it unlikely to be due to a bacterial infection. Simons and Peng named this clinical disorder Skeeter syndrome [20].

Mosquito Allergens

It has been suggested that there may be differences in the allergenic composition of mosquito saliva, salivary glands and whole body extracts. Mosquito saliva contains allergens that sensitize atopic individuals [64]. Sixteen IgE-reactive proteins with a molecular weight of between 16 and 95 kDa have been detected in the saliva and salivary glands of 10 different mosquito species distributed worldwide [44]. When analyzed with sera from allergic individuals, allergens in the saliva of *C. quinquefasciatus* with a molecular weight of 35.5, 32.5 and 22.5 kDa showed a frequency of IgE reactivity of 41, 28 and 24%, respectively [67]. Fourteen IgE reactivity fractions with a molecular weight of between 23 and 93 kDa were identified using sera from allergic patients in extracts from the salivary glands of *Aedes togoi*, *Culex tritaeniorhynchus* and *C. pip-*

iens pallens. In addition, mice immunized with these extracts produced mainly IgG1 that recognized bands of 31, 37 and 57.5 kDa [17], respectively, suggesting that these antigens could play a protective immune response.

Several studies have shown that mosquito saliva is not the only source of allergens. In fact, extracts used for allergen-specific immunotherapy and diagnosis are produced from saliva, salivary glands or whole mosquito bodies. Although these preparations have different biological activities, all are useful for the diagnosis of allergic individuals [68]. In Yazd, Iran, a subtropical city with a warm, dry climate, 32.6% of 95 patients with allergic rhinitis had a positive skin test to whole-body mosquito extracts. The frequency of reactivity to mould, cockroach and mite extracts was lower [24]. These results suggest that the antigens contained in mosquito bodies are important environmental factors associated with allergic rhinitis in this region. An extract prepared from airborne particles collected in the homes of mosquito-allergic patients could block the binding of IgE antibodies to the antigens contained in a whole-body extract of *C. quinquefasciatus* [18]. This study proposes that mosquito allergens are present in the environment and that they retain their immunological properties, as shown in ELISA inhibition experiments. When inhaled, these allergens can induce an allergic response in atopic individuals sensitized to mosquito bites. The clinical manifestations of this response are different from those caused by insect stings [69]. Similar findings have been described in south-eastern Minnesota, where large amounts of outdoor *Lepidoptera* allergens have been detected, suggesting that the inhalation of insect debris could play an important role in the ethiopathogenesis of allergic respiratory diseases [54]. Other biting insects such as fleas are also a source of indoor allergens, but their role as inducers of allergic diseases is less known [70]. Mosquito saliva primarily induces skin reactions, while the antigens that are suspended in the air may induce respiratory symptoms. Therefore, it is important to identify and characterize the allergens associated with these different clinical manifestations, in order to design better strategies for diagnosis and treatment. Allergen cross-reactivity must also be fully delineated to the contribution of species-specific or cross-reacting allergens.

We have demonstrated that the sera from mite-allergic individuals contain specific IgE antibodies that react with more than 10 allergens from an *A. aegypti* extract [71]. Although the clinical relevance of these allergens in allergic symptoms is unknown, the results suggest that mosquitoes are a source of aeroallergens, which may play a role in allergic respiratory diseases. Wongkamchai et al. [19] reported the presence of 16 allergens in the saliva, 6 in the salivary glands and 2 in whole-body extract of 4 mosquito species. These allergens are not common to all three sites, suggesting that allergic symptoms are related to the route of exposure (i.e. cutaneous or respiratory) and are associated with unique allergens. It is still necessary to further investigate if the allergens present in the saliva can induce respiratory symptoms.

Although several allergens have been identified in mosquito extracts, only a few from the saliva have been characterized in terms of their role in the allergic response. Most reported mosquito allergens belong to *A. aegypti* spp. (table 2) (see http://www.allergome.org and http://www.allergen.org).

Characterized Allergens

Aed a 1

This allergen shows homology with apyrase (ATP diphosphohydrolase) and has a molecular weight of 68 kDa. It interferes with platelet aggregation in human blood by hydrolyzing ADP and ATP released by the platelets and other cells [72]. It also has homology with members of a family of enzymes with 5′-nucleotidase activity, also present in humans and rats [73]. The serum of mosquito-allergic individuals may have specific IgE against this apyrase [44]. The truncated Aed a 1 (residues 150–562) was found to react with serum specific IgE and IgG antibodies of allergic individuals, suggesting that this fragment contains B cell epitopes [74]. In another study, 9 of 31 sensitized individuals (29%) had a positive skin test to rAed a 1 [15]. Rabbits immunized with Aed a 1 produced polyclonal sera that reacted with allergens in the saliva extracts of *A. vexans* and *Aedes albopictus* [44]. Apyrase activity was detected in the salivary glands of *Ochlerotatus triseriatus* and *Ochlerotatus hendersoni* [75], suggesting the presence of homologous allergens to Aed a 1. Because apyrase from mosquitoes inhibits platelet aggregation induced by collagen and thrombin, it has been suggested that it could be of therapeutic use in treating thrombosis [9]. It seems that Aed a 1 is expressed only in the salivary glands, which suggests that individuals may come into contact with this molecule through mosquito bites. However, it has still to be established whether this allergen can be detected in the environment and if it is capable of inducing sensitization through inhalation.

Aed a 2

This allergen belongs to the family of proteins called D7, which are highly conserved in insects that require blood for feeding and reproduction and are released in

Table 2. Reported *A. aegypti* allergens

Allergen	Molecular weight, kDa	Biological function	Amino acids, n	Frequency of reactivity[a] (patients, n/% positives)		References
				IgE	antigenic challenge	
Aed a 1	68	antiplatelet aggregant (apyrase)	562	–	31/29; 28/43	[15, 44, 74–76, 80]
Aed a 2	37	transport of hydrophobic molecules (pheromone and odorant)	321	21/42.8	28/11	[16, 44, 78–80]
Aed a 3	30	antiplatelet aggregant (aegyptin)	273	–	28/32	[81, 82]
Aed a 4	67	not described	579	25/36	–	[83]
Aed a 7	32.3	muscle contraction (tropomyosin)	284	–	–	see Allergome

Other mosquito species may also contain homologous allergens, as described in the text.
[a] Based on serology (IgE) or skin test (antigenic challenge).

the saliva just after piercing the skin. Aed a 2 contains an N-terminal and a C-terminal domain, capable of binding to leukotrienes and biogenic amines that are released by an individual as a mechanism of defense against the mosquito bite [76]. The gene encoding this protein was first identified in a cDNA library constructed from the salivary glands of *A. aegypti*. Northern blot assays and in situ hybridization showed that the mRNA of the protein is only present in the salivary glands of female mosquitoes, suggesting that it plays an important role in blood feeding [77]. This molecule has been cloned and obtained as a recombinant protein by expression in insect cells infected with baculovirus. It retains the ability to bind to specific human IgE antibodies and induces skin reactivity in a similar way to the natural protein [78]. Mice immunized with rAed a 2 produced IgG1 and IgE antibodies capable of binding to the recombinant and natural allergens. Sensitized mice showed a positive skin test to the recombinant allergen, which suggests that this protein induces a Th2 response, characteristic of an allergic reaction. Aed a 2 cross-reacts with proteins from other species of *Aedes* (*A. vexans*, *A. albopictus*, *A. communis* and *A. togoi*), *C. quinquefasciatus* and *O. triseriatus* [44]. The saliva of *C. quinquefasciatus* contains allergens homologous to Aed a 2 [79]. These allergens are homologous with the family of proteins D7 and show high structural homology with the protein THP12 of the hemolymph of *Tenebrio molitor*, which is part of the family of pheromone-binding proteins and odorants that participate in the transport of hydrophobic molecules [80]. It seems that Aed a 2 should be included in preparations for the diagnosis and immunotherapy of mosquito allergy, since it has a significant frequency of IgE reactivity [44] and may participate in cross-reactivity among various species. Because recombinant Aed a 2 is biologically and immunologically similar to the native purified allergen, it could be used in the diagnosis and immunotherapy of mosquito allergies as well. This would overcome the difficulty of obtaining this molecule from the saliva. Aed a 1 and Aed a 2 seem to be expressed only in mosquito salivary glands.

Additional Allergens

The allergens Aed a 3 and Aed a 4 have been obtained by molecular cloning and expression in insect cells infected by baculovirus. Aed 3 is expressed as a 30-kDa protein which binds specifically to collagen. It prevents the interaction of collagen with physiological ligands, such as platelet glycoprotein IV, integrin $\alpha 2\beta 1$ and von Willebrand factor, blocking platelet aggregation induced by collagen [81]. This property suggests its potential use as an antithrombotic drug. It has been suggested that this allergen may also be valuable for the diagnosis of mosquito allergy. It has been shown that a mixture of rAed a 1, rAed a 2 and rAed a 3 can identify approximately 65% of mosquito-allergic patients [82]. It has been shown that 36% of *A. aegypti*-allergic individuals have specific IgE against Aed a 4, suggesting that its inclusion in vaccine preparations might be desirable [83]. Aed a 7 is recorded in the Allergome platform, as an allergen with homology to tropomyosin. However, no further information is currently available about its biological and structural properties.

Allergen Cross-Reactivity

Allergen cross-reactivity occurs when IgE antibodies induced by exposure to an allergen react with a different protein with structural homology to the antibody-inducing allergen. This phenomenon is common among allergens from mites and other arthropods [84, 85]. The sera from individuals allergic to mosquito bites react with several common proteins present in the extracts of *C. quinquefasciatus*, *A. aegypti*, *A. albopictus* and *Anopheles minimus*, with molecular weights between 35.5 and 75.4 kDa [19]. These results suggest that exposure to 1 species may induce sensitization to other species to which the individual has not been previously exposed. In the province of Manitoba in Canada, the most prevalent mosquito species and the main sensitizer is *A. vexans*. Individuals allergic to *A. vexans* also show skin test reactivity to 5 other species not reported in Manitoba.

Mosquito-allergic individuals in Shanghai in China also have specific IgE against *A. vexans* allergens, despite the fact that this species has not been reported in this region [43, 44]. It can be speculated that, in these cases, allergen-specific immunotherapy with allergen extracts prepared from a single mosquito species might be sufficient to confer protection against other species. However, this should be carefully analyzed because species-specific allergens may exist and participate in the sensitization process. Vaccine preparations lacking species-specific allergens might thus be ineffective. A component-based molecular diagnosis would be of great value in these cases.

Rabbits immunized with rAed a 1 produce serum antibodies that react with extracts from *A. vexans* and *A. albopictus* [44], indicating that these 2 species have homolog allergens to that which is present in *A. aegypti*. In the salivary gland of *O. triseriatus* and *O. hendersoni*, a protein with apyrase activity has been detected; this might be a homolog of Aed a 1 [75]. Serum from rabbits immunized with rAed a 2 also reacted with extracts of *C. quinquefasciatus*, *O. triseriatus* and several species of *Aedes* [16, 44], indicating the participation of this allergen in the cross-reactivity among these 3 species.

C. quinquefasciatus, *A. aegypti* and *A. stephensi* express D7 family proteins; 73.1% of sera with IgE reactivity to *C. quinquefasciatus* also have specific IgE antibodies to whole-body extracts of *A. aegypti* and *A. stephensi* [80]. The D7 family is highly conserved in many species of blood-sucking insects and mites (ticks), so it is possible that cross-reactivity among these species may involve this protein family.

Studies of cross-reactivity between mosquitoes and wasps, dust mites, cockroaches and shrimp are scarce. However, some individuals develop the so-called ‘wasp/mosquito syndrome’, which involves a cross-reactivity phenomenon between these species. Individuals afflicted by this syndrome have specific IgE against a 44-kDa hyaluronidase in mosquitoes and wasps [86]. The existence of cross-reactivity between chironomids and mosquitoes, based on the results of positive skin tests to *C. pipiens* and serum IgE reactivity to *A. communis*, has also been suggested in individuals allergic to chironomids [45]. A study conducted in Reykjavik, Iceland, showed that most individuals sensitized to *D. pteronyssinus* also had specific IgE against mites *(Lepidoglyphus destructor)*, shrimps and, to a lesser extent, cockroaches and mosquitoes [87]. There were virtually no mites in the dust samples collected from mattresses in the homes of these individuals [46], indicating an absence of exposure to mite allergens. The sensitization to mites in this population could therefore be attributed to exposure to cross-reactive allergens from mosquitoes or other arthropods.

Cross-allergenicity has important clinical and epidemiological implications, such as polysensitization to various sources of allergens in the same or different taxonomic groups. To circumvent this problem, a better diagnosis could be achieved by identifying the specific molecules involved in the allergic response, which may be performed using highly purified recombinant or natural allergens in order to define the IgE-binding profile of each patient. Component-resolved diagnosis would allow a component-resolved immunotherapy, in which only the allergens involved in sensitization could be administered and not irrelevant allergens or nonallergenic molecules [88].

Sensitization to arthropods seems to be a predisposing factor for the onset of cutaneous and allergic respiratory responses to mosquitoes. Cross-reactivity among mosquitoes and other arthropods has been suggested. However, the main allergens involved here have not been identified. In the tropics, mosquitoes and the mite species *D. pteronyssinus* and *B. tropicalis* are very common. Therefore, it is probable that cross-reactivity between these arthropods may have an impact on the prevalence and manifestations of allergic diseases. We have shown that 40% of allergic individuals from the tropical Caribbean island, Martinique, have specific IgE against *A. aegytpi*. Furthermore, ELISA inhibition assays showed that *A. aegypti* contains allergens that cross-react with *D. pteronyssinus* allergens [89]. Two tropomyosin isoallergens

(Uniprot code: Q17H75 and Q17H80) of 32 kDa purified from a whole-body mosquito extract reacted with 33% of the mosquito-positive sera. They also showed cross-reactivity with the shrimp allergens: nPen m 1 and rLit v 1 [71]. These results confirm that there is a high frequency of sensitization to *A. aegytpi* in mite- and shrimp-allergic individuals in Martinique. Species-specific as well as cross-reactive allergens, such as tropomyosin, are involved in IgE sensitization.

Allergen-Specific Immunotherapy

There are only a few studies on mosquito immunotherapy. The natural history of mosquito allergy suggests that after prolonged exposure to mosquito bites, desensitization may occur. This is accompanied by a decrease in the serum levels of specific IgE and an increase in specific IgG antibodies [21, 48]. The abundance of *A. communis* in Finland in the summer is accompanied by increasing levels of IgE, IgG1 and IgG4 in the population. The increase in IgG subclasses may also play a protective role [90]. These observations suggest that immunotherapy with mosquito extracts or recombinant allergens could induce a protective response against subsequent exposures. Several studies have shown that immunotherapy with allergen extracts is effective for treating mosquito allergy. In a group of 20 mosquito-allergic individuals with a positive skin test and positive specific IgE determinations to *A. communis*, the subcutaneous application of whole-body extract for 18 months induced the disappearance of the skin lesions as well as respiratory and eye symptoms that appeared after the mosquito bite. The treatment also reduced the need for antiallergic medication. These effects were not observed in the control group that did not receive immunotherapy. No adverse reactions occurred during the treatment [91]. A randomized, double-blind, placebo-controlled trial showed that immunotherapy with extracts of *C. quinquefasciatus* for 1 year in 40 patients with asthma and/or rhinitis improved allergic symptoms and decreased bronchial hyperreactivity [92]. In addition, there was a slight decrease in IgE levels accompanied by a significant increase in IgG4, which is indicative of effective immunotherapy. These changes were not observed in the placebo group [92]. Administration of mosquito extracts mixed with extracts of other insects has shown efficacy in treating allergy to various insects. In another double-blind, placebo-controlled study, the group of patients treated for 12 months with one or mixed extracts of cockroach, mosquito or fly, showed decreased skin test reactivity and lower IgE levels after the 12 months of immunotherapy. Moreover, skin test results, allergic symptoms and bronchial hyperreactivity were significantly lower in the group treated with the extract compared with the placebo group. The treatment induced significantly lower IgE and higher IgG4 levels than in the placebo group [93].

Conclusions

Mosquito allergy is distributed worldwide, but is especially common in tropical regions and in areas where mosquitoes are abundant. Few studies have investigated the epidemiology and immunological mechanisms implicated in mosquito allergy. Early efforts to identify mosquito allergens focused mainly on the saliva. It was assumed that the bites were the only source of exposure to their allergens. However, airborne mosquito allergens may also play a sensitizing role. Studies on the cross-reactivity among different species of mosquito and other sources of allergens are scarce. However, the few available studies suggest an important degree of cross-reactivity between mosquitoes and other arthropods. A small number of mosquito allergens have been obtained and characterized by molecular cloning. Two recombinant allergens from *A. aegypti*, Aed a 1 and Aed a 2, have been isolated and obtained as highly purified molecules. The rate of sensitization to these two allergens is often less than 50%, which suggests that other allergens may play an important role. Studies have shown that allergen immunotherapy with mosquito-allergenic extracts is effective. More research is needed to produce a more specific diagnosis and effective treatment for mosquito allergies.

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Disclosure Statement

The authors report no conflict of interests in relation to this paper.

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IV. HYPOTHESIS

Aedes aegypti contains salivary as well as somatic allergens. Somatic allergens may comprise species specific as well as cross-reactive allergens.

V. OBJECTIVES

A. Main objective

The main objective of this thesis was to study the allergenic components from the mosquito *Aedes aegypti* in the immune response in humans and mice

B. Specific objectives:

The specific objectives of this thesis were to:

1. Prepare whole body allergenic extracts from the mosquito species *A. aegypti* and analyse its total and IgE-binding protein spectrum using the sera from allergic individuals
2. Analyse the allergenome of *A. aegypti,* from its proteome, separated by 2D-electrophoresis
3. Purify natural IgE-binding tropomyosin from the whole body extract by high performance liquid chromatography.
4. Characterize the identified IgE-binding proteins by Mass spectrometry approaches.
5. Obtain at least two pure recombinant allergens by expression in bacteria and analyse their IgE-binding capacity
6. Study the folding and allergenicity of the recombinant mosquito allergens, by circular dichroism and basophil activation test, respectively.
7. Immunize mice with *A. aegypti* body extract and recombinant *A. aegypti* allergens
8. Analyse the antibody, cytokine and proliferative response of the mice immunized with *A. aegypti* allergens
9. Study the molecular and cellular cross-reactivity between *A. aegypti* and other arthropod-derived allergens, using the allergenic extracts and the purified allergens.

VI. RESULTS

A. Identification of novel IgE-binding proteins from the mosquito species *A. aegypti* using a proteomic approach

In this part of the project, a comprehensive identification and characterization of mosquito allergens was achieved by a combination of 2-dimensional electrophoresis, immunoblotting and mass spectrometry. Ten different proteins were identified and their clinical significance in allergy diagnosis explored. Five identified allergens were deposited in the WHO/IUIS allergen nomenclature database under the names of Aed a 5, Aed a 6, Aed a 7, Aed a 8 and Aed a 11.

The study of the role of mosquitoes in the pathophysiology of the allergic responses requires the identification of the IgE-binding molecules. Several approaches may be used for this purpose. We decided to apply proteomic tools that allowed the study of a large panel of molecules at the same time. The proteome and transcriptome of *A. aegypti* have been studied to discover potential receptors for insecticidal proteins, to understand the mechanisms of infection for some viruses, to study the molecular mechanisms associated with mosquito bites and find novel approaches to control the infestation of this mosquito. However, these tools had never been applied in the field of mosquito allergy research. The advantages of Proteomic sciences applied to allergy research is evidenced in studies with mites, fish, insects and chicken, leading to an important growth of the list of allergens from these species.

For further information see the following paper:

Article 2.

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ORIGINAL ARTICLE

Experimental Allergy and Immunology

Allergenome characterization of the mosquito *Aedes aegypti*

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Abstract

Background: Saliva and muscle-derived mosquito allergens have been purified and characterized. However, the complete set of allergens remains to be elucidated. In this study, we identified and characterized IgE-binding proteins from the mosquito species *Aedes aegypti*.

Methods: Serum was obtained from 15 allergic individuals with asthma and/or rhinitis and sensitized to mosquito. IgE binding was determined by ELISA. Total proteins from freeze-dried bodies of *A. aegypti* were extracted and IgE-reactive proteins were identified by 2D gel electrophoresis, followed by Western blot with pooled or individual sera. IgE-reactive spots were further characterized by mass spectrometry.

Results: Twenty-five IgE-reactive spots were identified, corresponding to 10 different proteins, some of which appeared as different variants or isoforms. Heat-shock cognate 70 (HSC-70) and tropomyosin showed IgE reactivity with 60% of the sera, lysosomal aspartic protease, and "AAEL006070-PA" (Uniprot: Q177P3) with 40% and the other proteins with <33.3% of the sera. Different variants or isoforms of tropomyosin, arginine or creatine kinase, glyceraldehyde-3-phosphate dehydrogenase (GPDH), calcium-binding protein, and phosphoglycerate mutase were also identified. The mixture of three allergens (Aed a 6, Aed a 8, and Aed a 10) seems to identify more than 80% of *A. aegypti*-sensitized individuals, indicating that these allergens should be considered when designing of improved mosquito allergy diagnostic tools.

Conclusions: The newly identified allergens may play a role in the pathophysiology of mosquito allergy in the tropics, and some of them might be important arthropod-related proteins involved in cross-reactivity between *A. aegypti* and other allergenic arthropods.

KEYWORDS
Aedes aegypti, allergen, allergenome, insect allergy, mosquito

1 | INTRODUCTION

Allergic respiratory diseases affect 10-30% of the population worldwide.[1,2] Several arthropods, including house dust mites (HDM), cockroaches and shrimps, participate in the onset and severity of allergic asthma, rhinitis, and other allergic diseases, such as food allergies.[3-5] Mosquitoes are worldwide distributed[6] and contain several IgE-binding proteins[7]; some of which cross-react with allergens from other arthropods.[8] *Aedes aegypti* is a mosquito that can spread dengue fever, chikungunya, Zika fever, yellow fever viruses, and other diseases.[9]

Aedes aegypti can also induce recurrent allergic cutaneous reactions upon insect bites.[7] Four saliva-derived allergens, Aed a 1 (apyrase), Aed a 2 (D7 family protein), Aed a 3 (salivary protein), and Aed a 4 (α-glucosidase), are currently registered in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature data set (http://www.allergen.org). Up to 65% of mosquito bite-allergic patients have specific IgE against a mixture of Aed a 1, Aed a 2, and Aed a 3.[10] We have previously reported a novel nonsalivary allergen, Aed a 10 (tropomyosin), which cross-reacts with other tropomyosins from several arthropods. IgE binding against Aed a 10 was demonstrated in allergic individuals with asthma and/or allergic rhinitis.[11] *Aedes aegypti* has additional IgE-binding proteins that remain to be characterized. This insect contains at least eight allergens in the saliva[12] and Western blot studies with body extract revealed at least 12 allergens reacting with the sera from individuals with asthma, or allergic rhinitis. The knowledge of the complete repertoire of allergens from *A. aegypti* is limited, and further efforts for the identification and characterization of these molecules are warranted.

In this study, we analyzed the allergenome of *A. aegypti* by 2D electrophoresis and the combination of immunoblotting and MS. The frequency of IgE binding of each protein was determined by immunoblotting with individual sera.

2 | MATERIALS AND METHODS

2.1 | Serum samples

Serum was obtained from 15 allergic patients who attended an outpatient Allergy Clinic in Fort de France, Martinique (Table 1). A serum pool was prepared mixing equal volume of seven sera (# 3, 5, 8, 12, 13, 14, and 15), in order to have a more complete representation of specific IgE to the tested allergens. Serum was also obtained from nonallergic individual with total IgE of 47 kU/L. All individuals were skin-tested with a panel of common standardized inhalant allergens and with a commercially available *Aedes communis* extract (Stallergènes, Antony, France). Individuals who developed a wheal >3 mm to the extracts were considered to have a positive skin prick test (SPT). Serum was obtained after written consent in the context of the approved study entitled: "Etude Epidémiologique des causes Allergiques de l'asthme en Martinique," funded and approved by "Fonds de'aide a la qualite de soins de ville" from "la Caisse Generale de securite sociale de la Martinique" and the "Comite departemental de la Martinique contre les maladies respiratoires et la tubreculose (CDMMRT)."

2.2 | Total protein extraction

Defatted and lyophilized *A. aegypti* mosquito bodies material was purchased from GREER laboratories (Lenoir, NC, USA). Total protein was extracted with ReadyPrep™ Protein Extraction Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Briefly, 200 mg of mosquito were mixed with 2-D rehydration buffer, sonicated and centrifuged and the supernatant further treated with ReadyPrep 2-D Cleanup Kit (Bio-Rad) following manufacturer's instructions. Briefly, sample was incubated with precipitating reagent 1 followed by precipitating reagent 2. The precipitate was washed with wash reagent 1. The pellet was mixed with milliQ water and incubated with wash reagent 2. The pellet was air-dried and

TABLE 1 Clinical details and sensitization profiles of 15 allergic individuals used in the study

Serum	Age (years)	Gender	Diagnosis	Total IgE IU/mL	Specific IgE against *Aedes aegypti* (O.D.)
1	4	M	Asthma, rhinitis, conjunctivitis	1659.0	0.511
2	5	F	Asthma	1474.0	0.248
3	4	M	Asthma, conjunctivitis	2187.0	0.458
4	4	F	Rhinitis, conjunctivitis	2604.0	1.221
5	11	M	Asthma, rhinitis, conjunctivitis	2095.4	1.679
6	6	M	Rhinitis, conjunctivitis	552.00	0.513
7	4	F	Asthma, rhinitis	429.58	1.111
8	27	F	Rhinitis	545.50	0.355
9	3	M	Rhinitis	2853.0	1.982
10	5	F	Asthma, rhinitis, conjunctivitis	591.00	0.485
11	3	F	Asthma, rhinitis	1615.0	0.573
12	6	M	Rhinitis, conjunctivitis	647.00	0.501
13	31	M	Rhinitis	97.400	0.382
14	9	M	Asthma	1260.0	0.379
15	6	M	Rhinitis	324.00	0.319
Mean	8.53	60% M		1433.3	0.714

F, female; M, male.
O.D.: optical density, measured by ELISA.
All patients developed a wheal diameter >3 mm after skin prick test with extracts of *Aedes communis*, *D. pteronyssinus*, *D. farinae*, *Blomia tropicalis*, shrimp, and cockroach.

resuspended with ReadyPrep 2D rehydration/sample buffer (8 mol/L urea, 2% CHAPS, 50 mmol/L DTT, 0.2% Bio-Lyte 3/10 ampholyte, 0.001% bromophenol blue). Protein concentration was measured using RC DC Protein Assay (Bio-Rad).

2.3 | 2D electrophoresis

Isoelectric focusing (IEF) was performed using 11 cm, pH 3-10 linear IPG strips (Bio-Rad). Strips were rehydrated with 200 μL of rehydration buffer containing 200 μg of protein, at room temperature. Proteins were focused at 50 μA/IPG strip for a total of 26 000 V/h at 20°C using the PROTEAN i12 IEF System (Bio-Rad). After focusing, the strips were washed 10 minutes in equilibration buffer 1 containing 6 mol/L urea, 20% v/v glycerol, 2% w/v SDS, 0.375 mol/L Tris-HCl (pH 8.8), and 2% DTT, followed by equilibration buffer 2 containing 6 mol/L urea, 20% v/v glycerol, 2% w/v SDS, 0.375 mol/L Tris-HCl (pH 8.8), and 2.5% w/v iodoacetamide. Afterward, IPG strips were washed with SDS-PAGE running buffer (25 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS) and applied onto the top of 12.5%, 11 cm, Criterion TGX Precast Gels (Bio-Rad). Strips were covered with ReadyPrep Overlay Agarose (Bio-Rad) and electrophoresis performed at 200 V for 65 minutes. Gels were stained with GelCode Blue stain reagent (Life Technologies, Carlsbad, CA, USA). Precision Plus Protein Standard Plugs (Bio-Rad) were used to determine the molecular weights.

2.4 | SDS-PAGE, IgE immunoblot and inhibition analysis

Proteins from *A. aegypti* were separated by one-dimensional electrophoresis. Proteins from the one-dimensional and two-dimensional gel electrophoresis were electrotransferred to nitrocellulose membranes and IgE binding evaluated using the serum pool or individual sera as described in.[11]

For the inhibition experiments, proteins from the one-dimensional gel electrophoresis were electrotransferred to nitrocellulose membranes and incubated with sera 1 and 5, previously adsorbed with 10 μg/mL of rAed a 10.0101 or rAed a 10.0201, obtained as described in.[11] Specific IgE binding was detected as described previously.

2.5 | Selection of specific IgE binding spots

Coomassie-stained and immunoblotted 2D gels were compared using GeneTools image analysis software (Synoptics, Cambridge, UK). Each immunoreactive spot was located on the image of the corresponding immunoblot and Coomassie-stained gel. The molecular mass and isoelectric point (IEP) of each spot were calculated by comparison with the standard and the IEP gradient. Each spot detected in the immunoblot was compared with a grid after alignment with the Coomassie-stained gel. Spots that matched with similar molecular weight and IEP were further characterized by MS.

2.6 | MS analysis

Spots of interest from 2DE were excised and analyzed using either peptide mass fingerprint (PMF) or peptide fragment fingerprint (PFF); at the CIC bioGUNE, Derio, Spain.

2.7 | Tryptic digestion

Gel spots were washed with 50 mmol/L ammonium bicarbonate, incubated with DTT (10 mmol/L in ammonium bicarbonate) at 56°C for 20 minutes, followed by iodoacetamide (50 mmol/L in ammonium bicarbonate) for 20 minutes in the dark. Spots were dried and incubated with trypsin (12.5 μg/mL, in ammonium bicarbonate, 10 μL) for 20 minutes on ice. After rehydration, the supernatant was discarded. Gel pieces were incubated overnight at 37°C. After digestion, acidic peptides were extracted with 0.1% TFA and dried in a RVC2 25 Speedvac concentrator (Christ, Germany).

2.8 | PMF analysis

Peptide mass fingerprint analysis was performed using matrix-assisted laser desorption time of flight (MALDI-ToF/ToF) equipment in a MALDI-LIFT-ToF AUTOFLEX III Smartbeam (Bruker Daltonics, Billerica, MA, USA). One microliter of digested sample was loaded onto a target plate (Bruker 384 ground steel) with 1 μL of HCCA matrix. Data-dependent MS acquisitions were performed with charge state of 1 over a survey m/z range of 500-4000. Ionization was performed with a solid-state laser of 360 nm and 200 Hz. Laser intensity energies were varied depending on the analysis required. For MS, 30-50% of intensity was used and around 90% for MS/MS. Resolution was always over 7500 along all mass-window range for MS analysis. Data acquisition was performed manually. Routinely 1400 scans were collected for PMF, whereas the most intense peaks were selected for MS/MS (400 scans for parent selection and 1600 scans for fragments). Calibration was performed externally, with pepmix (Bruker Daltonics), and internally, with trypsin peptides.

The spectra obtained were processed using Flex analysis 3.0 and Biotools 3.2 (Bruker Daltonics). Database searching was performed using MASCOT 2.2.07 (Matrixscience, London, UK) against a custom database filled with all the Uniprot entries corresponding to *A. aegypti* (16 654 sequences; 7 684 696 residues). For identifications where one peptide was assigned to protein, data were researched against SwissProt (Uniprot) (546 439 sequences; 194 445 396 residues), and NCBI (51 471 198 sequences; 18 460 256 539 residues) databases to confirm the identification. In all searches, for protein identification the following parameters were adopted: carbamidomethylation of cysteine (C) as fixed modification and oxidation of methionine (M) as variable modifications, 50 ppm of peptide mass tolerance, 0.7 Da fragment mass tolerance and up to two missed cleavage points.

Spots not identified by PMF, or identified with poor confidence were re-analyzed by PFF using nano-liquid chromatography-tandem mass spectrometry (nLC-MS/MS).

2.9 | PFF analysis

Separation of tryptic peptides was performed using NanoAcquity nano-LC (Waters Corporation, Milford, MA, USA) equipment, on a Waters BEH C18 nano-column (200 mm × 75 μm ID, 1.8 μm). Peptide elution was performed using a chromatographic ramp from 5% to 60% of mobile phase B during 30 minutes, and flow rate of 300 nL/min. Mobile phase A was water containing 0.1% v/v formic acid, while mobile phase B was acetonitrile containing 0.1% v/v formic acid. MS data were obtained on a Synapt G2Si ESI Q-Mobility-TOF spectrometer (Waters) equipped with an ion mobility chamber (T-Wave-IMS) for high-definition data acquisition analyses. All analyses were performed in positive ESI mode. A lock mass compound [Glu1]-Fibrinopeptide B (100 fmol/μL) was delivered by an auxiliary pump of the LC system at 500 nL/min to the reference sprayer of the NanoLockSpray (Waters) source of the mass spectrometer. Data were postacquisition lock mass-corrected using the double charged monoisotopic ion of [Glu1]-Fibrinopeptide B. Accurate mass LC-MS data were collected in HDMS mode which enhances signal intensities using the ion mobility separation. Database searching was performed using MASCOT 2.2.07 (Matrixscience) against a custom database filled with all the UNIPROT entries corresponding to *A. aegypti* (16 654 sequences; 7 684 696 residues). For protein identification, the following parameters were adopted: carbamidomethylation of cysteine (C) as fixed modification and oxidation of methionine as variable modifications, 10 ppm of peptide mass tolerance, 0.5 Da fragment mass tolerance and up to 1 missed cleavage points, and peptide charges of +2 and +3.

2.10 | Allergenicity test and bioinformatic analysis

Frequency of IgE binding for each spot was determined in the immunoblots with all individual sera. Bioinformatics analysis to predict potential allergenicity were performed using: (i) Allertop V.2 (http://www.ddgpharmfac.net/AllerTOP)[13] which is based on amino acid descriptors, accounting for residue hydrophobicity, size, abundance, helix and β-strand forming propensities, (ii) PREAL (http://gmobl.sjtu.edu.cn/PREAL/index.php),[14] which integrates various properties of proteins, such as biochemical and physicochemical properties, sequential features and subcellular locations, and (iii) Algpred (http://www.imtech.res.in/raghava/algpred/)[15] which predicts based on similarity of known epitope with any region of protein.

3 | RESULTS

3.1 | Proteomic identification of IgE-reactive proteins

The proteome profile of *A. aegypti* was obtained by 2D electrophoresis in the IEP range of 3-10 (Figure 1A). Specific IgE immunoblots were performed after incubation with the serum pool (Figure 1B). Twenty-five IgE immunoreactive spots were selected and further analyzed by MS. Immunoblot of a 2D gel with serum from one nonallergic individual showed no IgE binding (Figure 1C).

Twenty spots were characterized by PMF. Sixteen belonged to *A. aegypti* and showed significant score after Uniprot database searching. Four proteins showed homology with proteins from *A. aegypti* and other insects. Their identities were confirmed after Swiss-Prot or NCBI database searching. MS/MS spectra were acquired from the 20 spots, and identification was further confirmed with the deduced peptide sequence. Five spots that were not characterized by PMF were subjected to PFF analysis. Identification was achieved after database searching against Uniprot.

The 25 spots corresponded to 10 different proteins. Spot nos. 6-12, 13-17, 19-20, and 21-23 consisted of isoforms or variants with similar molecular weight and different IEP (Table 2 and Figure 1). The sequences of these proteins were previously inferred from genes that were identified in the *A. aegypti* genome and deposited in the Uniprot database.[16] Except for tropomyosin,[11] their natural counterparts have not been previously identified, purified, or studied.

Among the identified IgE-reactive proteins, tropomyosin was the only previously known allergen, named as Aed a 10, with two variants (Aed a 10.0101 and Aed a 10.0201).[11] Two spots (no. 1 and no. 5) corresponded to lysosomal aspartic protease and heat-shock cognate 70 (HSC-70), respectively. Other spots corresponded to proteins with ATP-binding activity (no. 4), calcium ion binding activity (no. 18), phosphoglycerate mutase activity (nos. 21-23), and voltage-dependent anion-selective channel activity (no. 24). Variants or isoforms of “arginine or creatine kinase” (Uniprot code: Q1HR67) (nos. 6-12) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) (Uniprot code: J9HYM2) (nos. 13-17). A hypothetical protein (Uniprot code: Q16TN9) was also identified (no. 25).

3.2 | Relevance of tropomyosin family in the IgE-binding protein repertoire of *Aedes aegypti*

In order to further study the relevance of tropomyosin family in the IgE-binding protein repertoire, we performed an immunoblotting inhibition with 2 individual sera, using rAed a 10.0101 and rAed a 10.0201 as inhibitors. Pre-incubation with rAed a 10.0101 inhibited the IgE binding of the 32 kDa band, which represent the native mosquito tropomyosin. rAed a 10.0201 produced partial reduction of the IgE binding. No changes in the IgE-binding capacity to more than 10 different bands were detected (Figure 1D).

3.3 | Frequency of specific IgE binding

A variable pattern of IgE binding among the individual sera was observed (Figure 2). We classified a protein as “major allergen” if it reacted with >50% of sera.[17] Tropomyosin and HSC-70 could be major allergens, as they reacted with 60% of the sera (Table 3). Lysosomal aspartic protease and “AAEL006070-PA” (Uniprot code: Q177P3) reacted with 40% of the sera. The other six proteins reacted with <26.7% of the sera. “Arginine or creatine kinase” and

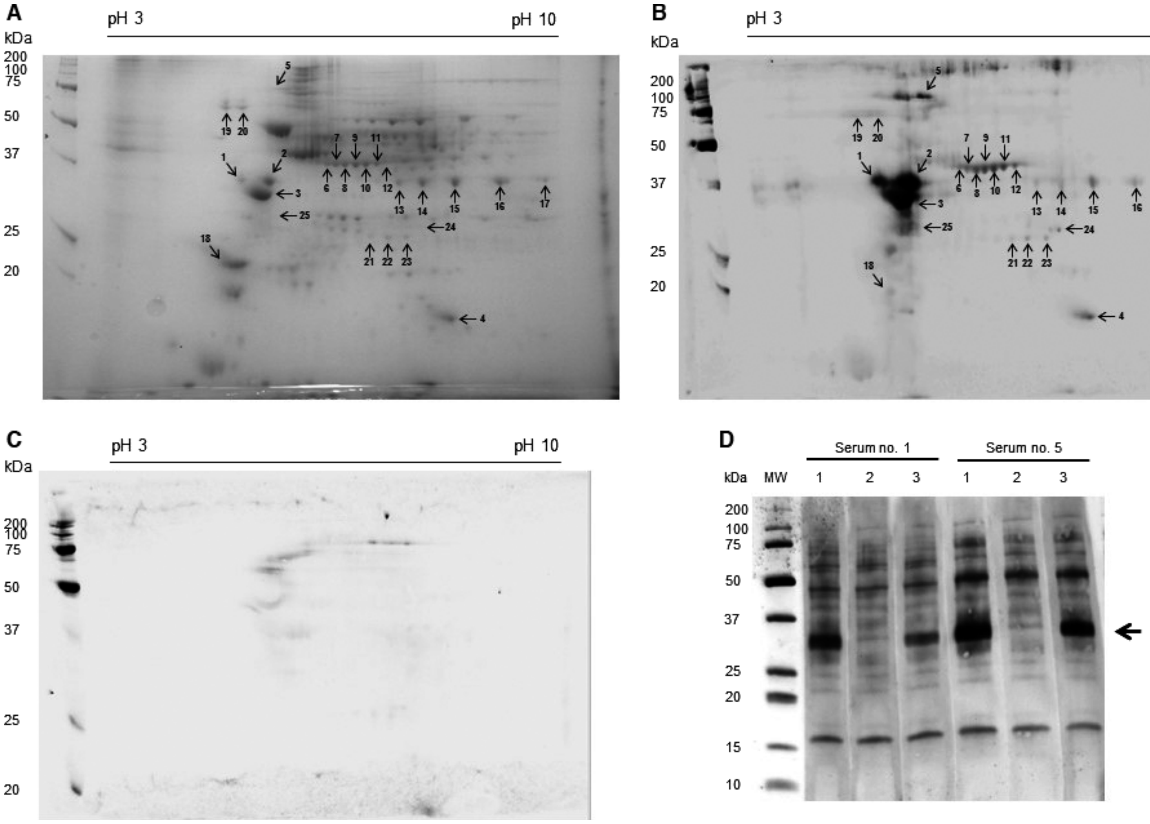

FIGURE 1 Two-dimensional gel electrophoresis of total proteins from *Aedes aegypti*. (A) Spots were identified by Coomassie stain, and (B) specific IgE immunoblot using pooled sera. (C) Serum from a nonallergic individual did not show any significant binding. The proteins showing immunoreactivity are marked with arrows. Immunoblotting indicating IgE binding of *A. aegypti* extract (D1) and inhibition assays using rAed a 10.0101 (D2) or rAed a 10.0201 (D3) as inhibitors. Adsorption of sera with 10 μg/ml of recombinant tropomyosins abrogated the reactivity

GPDH had a lower frequency of IgE binding (13.3% and 6.7%, respectively). However, the intensity of reactivity to the isoforms or variants of GPDH was different. Spots 14, 15, and 16 were those with the highest color intensity. No IgE binding was detected with the control serum.

Based on the frequency of IgE binding, it seems that there are no immunodominant allergens in *A. aegypti*. The combination of the two tropomyosin variants (Aed a 10.0101 and Aed a 10.0201) and HSC-70 recognized IgE binding in 11 of 15 patients (73.33%). Adding Aed a 6 (spot no. 24), the percentage increased to 86.7% (Table 3).

3.4 | Novel *Aedes aegypti* allergens

The WHO/IUIS Allergen Nomenclature Subcommittee (www.allergen.org) has assigned official names to five proteins identified in this study: Aed a 11.0101 for Q03168 (lysosomal aspartic protease), Aed a 5.0101 for Q16XK7 (sarcoplasmic Ca+ (EF-hand) binding protein), Aed a 6.0101 for Q1HR57 (Porin 3), Aed a 7.0101 for Q16TN9, and Aed a 8.0101 for Q1HR69 (HSC-70) (Table 4).

Bioinformatic analysis using Allertop, PREAL, and ALGPRED predicted that Aed a 8 is an allergen (Table 3). Multiple sequence alignment between Aed a 8 and HSP-70 protein from *Dermatophagoides pteronyssinus*,[18] *Cladosporium herbarum*,[19] and *Penicillium citrinum* [20] showed that the overall sequences are highly conserved (Data not shown). Aed a 5 was predicted as an allergen by Algpred and PREAL and Aed a 6 by Allertop and Algpred (Table 3).

4 | DISCUSSION

The proteome and transcriptome of *A. aegypti* have been studied to: (i) discover potential receptors for insecticidal proteins,[21,22] (ii) understand the mechanisms of infection for some viruses,[23,24] (iii) study the molecular mechanisms associated with mosquito bites,[25,26] and (iv) find novel approaches to control the infestation of this mosquito.[27] However, to the best of our knowledge, the present study is the first that uses proteomic tools to contribute to the understanding of the role of *A. aegypti* in allergic diseases, by the elucidation of its allergenic components. The proteomic approach used represents an accurate and high-resolution procedure to identify new allergens, which has been useful to identify food allergens[28,29] and allergenic components from *Dermatophagoides farinae*,[18] *Aspergillus fumigatus*,[30] insect venom,[31] and cockroach.[32]

The manifestation of mosquito allergy is a matter of controversy. We hypothesize that mosquito allergens are distributed in the body

TABLE 2 Protein identification of specific IgE-binding proteins from *Aedes aegypti*

Spot no.	Accession number	Protein name	Previous allergen name in IUIS	Function	Mascot score	Second Mascot score	Coverage (%)	Matched peptides/total peptides observed	Experimental MW/pI	Theoretical Mw/pI[a]
1	Q03168[b]	Lysosomal aspartic protease	N.A.	May degrade organelles	310	76	26	–	32.6/5.2	41.8/5.2
2	Q17H75[b]	Tropomyosin	Aed a 10.0101	Muscle contraction	1783	834	70	–	32.0/5.5-5.7	32.7/4.7
3	Q17H82 or Q17H80	Tropomyosin	Aed a 10.0201 for Q17H80	Muscle contraction	207 or 194	51	34	10/32	30.2/5.3-5.6	32.4/4.8
4	Q16QR7[b]	AAEL011197-PA	N.A.	ATP binding[c]	137	73	22	–	15.0/8.2-8.3	41.2/5.3
5	Q1HR69	Heat-shock cognate 70	N.A.	Stress response ATP binding[c]	120	34	16	9/37	69.0/5.6	72.3/5.1
6	Q1HR67	Arginine or creatine kinase	N.A.	Kinase ATP binding[c]	144	42	12	3/6	36.0/6.3	39.9/6.0
7					226	44	14	4/4	35.8/6.4	
8					129	31	10	3/4	35.7/6.6	
9					107	18	10	3/31	35.4/6.7	
10					72	32	8	2/7	35.5/6.9	
11					138	35	16	4/8	35.5/7.0	
12					102	33	8	2/10	35.7/7.2	
13	J9HYM2	Glyceraldehyde-3-phosphate dehydrogenase	N.A.	Catalytic activity NAD binding[c]	123[d]	38	4	1/6	32.5/7.5	35.4/8.3
14					115[d]	29	4	1/18	32.1/7.9	
15					178	33	16	3/8	32.1/8.3	
16					171	30	23	4/25	32.0/9.0	
17					104[d]	33	4	1/8	32.3/9.7	
18	Q16XK7[b]	AAEL008844-PA	N.A.	Calcium ion binding[c]	938	58	72	–	17.9/4.9	21.9/4.6
19	Q17H75 or Q17H76[b]	Tropomyosin	Aed a 10.0101 for Q17H75	Muscle contraction	465	72	41	–	56.9/4.9	32.7/4.7
20					623	286	42	–	56.9/5.2	
21	Q177P3	AAEL006070-PA	N.A.	Phosphoglycerate mutase activitiy[c]	99[e]	26	3	1/8	24.8/7.1	28.5/6.3
22					139	34	21	5/20	24.7/7.4	
23					98	44	12	4/32	24.5/7.6	
24	Q1HR57	AAEL001872-PA	N.A.	Voltage-dependent anion-selective channel activity[c]	86	34	12	2/3	24.7/7.7	30.7/8.63
25	Q16TN9	AAEL010180-PA	N.A.	Unknown	98	33	12	3/28	27.4/5.4	23.5/4.7

N.A., Not previous allergen name assigned in IUIS.

[a] Theoretical MW and pI was assigned using Compute MW/pI tool available on ExPASy.

[b] Protein identified using nLC-MS/MS.

[c] Information from Gene Ontology.

[d] Identity confirmed by searching data against SwissProt database.

[e] Identity confirmed by searching data against NCBI database.

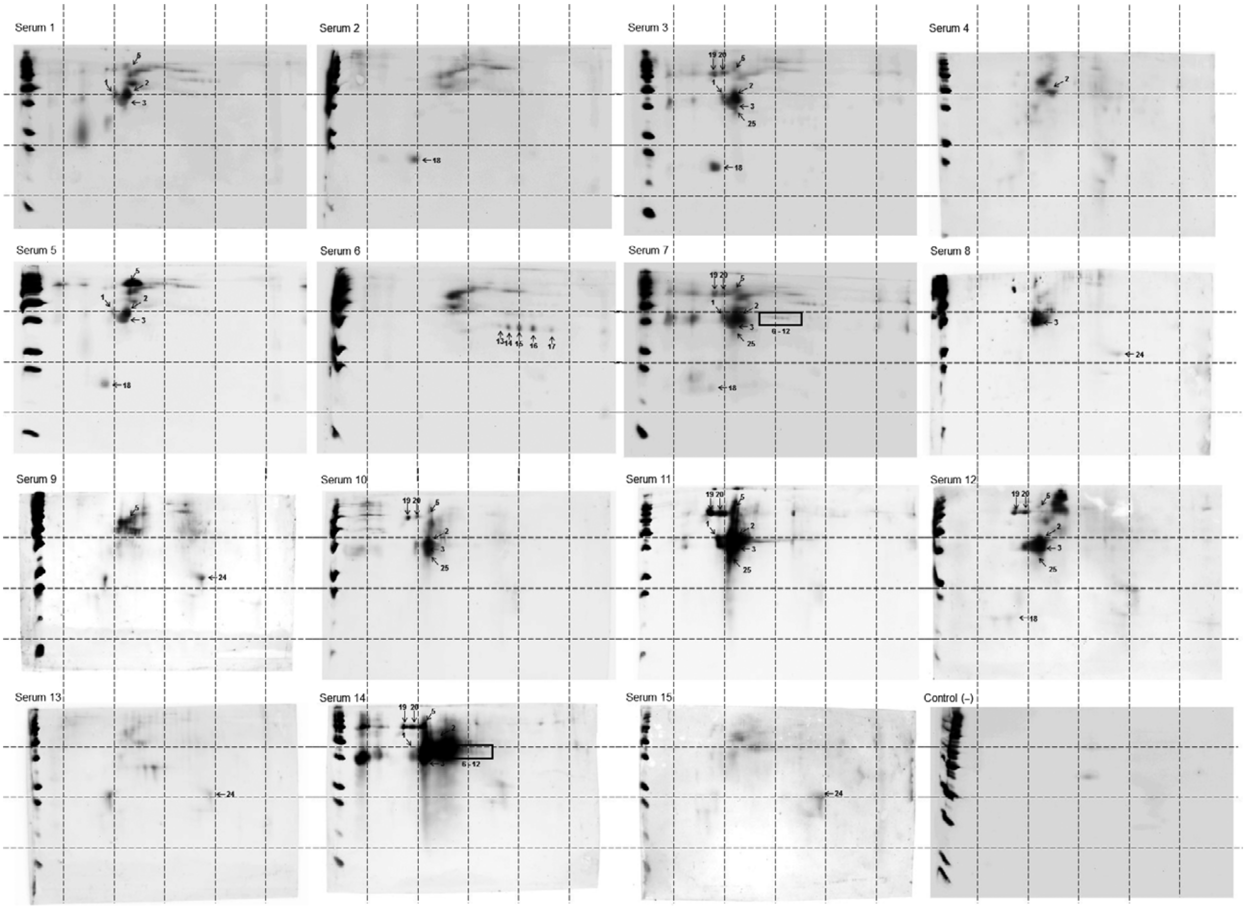

FIGURE 2 Specific IgE immunoblot of total proteins from *Aedes aegypti*, using individual sera. The immunoblots correspond to patient numbers 1-15. The proteins showing immunoreactivity are marked with arrows and boxes

TABLE 3 Allergenicity of *Aedes aegypti* components based on the serum IgE reactivity in 15 individuals with respiratory allergies

		Patient																
Spot	Protein name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Frequency of reactivity (%)	Predicted as allergen by
1	Lysosomal aspartic protease	+		+		+		+				+			+		40	Nonevaluated
2	Tropomyosin	+		+	+	+		+			+	+	+		+		60	Allertop, PREAL, ALGPRED
3	Tropomyosin	+		+		+		+	+		+	+	+		+		60	Allertop, PREAL, ALGPRED
4	AAEL011197-PA																0	Nonevaluated
5	HSC-70	+		+		+		+		+	+	+	+		+		60	Allertop, PREAL, ALGPRED
6-12	Arginine or creatine kinase							+							+		13.3	Nonevaluated
13-17	GPDH						+										6.7	Nonevaluated
18	AAEL008844-PA		+	+		+		+									26.7	PREAL, ALGPRED
19-20	Tropomyosin			+				+			+	+	+		+		40	Nonevaluated
21-23	AAEL006070-PA																0	Nonevaluated
24	AAEL001872-PA								+	+				+		+	26.7	Allertop, ALGPRED
25	AAEL010180-PA							+			+	+	+				26.7	Nonevaluated

and not only in the saliva, as patients with respiratory tract allergic reactions have specific IgE against body components.

The genome of *A. aegypti* contains 11 genes which codify for tropomyosin isoforms and variants.[16] We have previously reported the presence of four of these tropomyosins.[11] Two of them correspond to Aed a 10.0101 and Aed 10.0201 allergens. Spot no. 2 was identified as Aed a 10.0101, and the data suggest that spot no. 3 corresponds to tropomyosins with Uniprot codes Q17H80 and Q17H82. We were not able to differentiate these variants as no unique peptides were obtained. However, we hypothesize that spot

TABLE 4 Summary of peptides from the five new allergens of *Aedes aegypti*, identified by mass spectrometry analysis

Spot	Protein name	Allergen name	Identified peptides	Peptides specific for *A. aegypti*	Peptides specific for *A. aegypti* and other mosquito species
1	Lysosomal aspartic protease	Aed a 11	DPSAAEGGEIIFGGSDSNK QTFAEAINEPGLVFVAAK SFDLEGADYVLR YTGDFTYLSVDRK ECSFTNIACLMHNK VVFDTGSSNLWVPSK YTGDFTYLSVDR YYTEFDMGNDR YYTEFDMGNDR	SFDLEGADYVLR	DPSAAEGGEIIFGGSDSNK YTGDFTYLSVDRK ECSFTNIACLMHNK YTGDFTYLSVDR
5	HSC-70	Aed a 8	FDLTGIPPAPR DVDEIVLVGGSTR AKFEELNMDLFR ITPSYVAFTADGER NQLTTNPENTVFDAKR VTHAVVTVPAYFNDAQR DNHLLGKFDLTGIPPAPR KVTHAVVTVPAYFNDAQR IEIESFYEGDDFSETLTR		IEIESFYEGDDFSETLTR
18	AAEL008844-PA	Aed a 5	VDDSYNQLVSDEDNK AFIDAHYQMMDINNDGLVSIEEYR YQELYAQFMGNESDK KVDDSYNQLVSDEDNK ALWDEISALADLDHDGK GGITLQR VDDSYNQLVSDEDNKR SYSDFPK IAMDDIK LNEYR	VDDSYNQLVSDEDNK KVDDSYNQLVSDEDNK ALWDEISALADLDHDGK VDDSYNQLVSDEDNKR	AFIDAHYQMMDINNDGLVSIEEYR YQELYAQFMGNESDK
24	AAEL001872-PA	Aed a 6	EFGGLIYQR VTANNFALGYSTGDFVLHTNVNDGR	VTANNFALGYSTGDFVLHTNVNDGR	
25	AAEL010180-PA	Aed a 7	FIQNELPR ETLVTLLLR FVLLHEFIR	ETLVTLLLR FVLLHEFIR	

2DE, two-dimensional gel electrophoresis; ACN, acetonitrile; BSA, bovine serum albumin; CDMMRT, Comite departemental de la Martinique contre les maladies respiratoires et la tubreculose; ELISA, enzyme-linked immunosorbent assay; ESI, Electrospray ionization; GPDH, glyceraldehyde-3-phosphate dehydrogenase; HCCA, alpha-Cyano-4-hydroxycinnamic acid; HDM, house dust mites; HSC-70, heat-shock cognate 70; HSP70, heat-shock protein-70; kDa, kilo Dalton; MALDI, matrix-assisted laser desorption time of flight; MS, mass spectrometry; nLC, nano-liquid chromatography; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; ToF, time of flight; WHO/IUIS, World Health Organization and International Union of Immunological Societies.

no. 3 corresponds to Aed a 10.0201 (Q17H80). Proteins on spot nos. 19 and 20 appear with apparent molecular weights of 56.9 kDa and IEP of 4.9 and 5.1. Peptide information suggests that they correspond to tropomyosin Q17H75, or Q17H76. According to this information, we believe that it could correspond to a dimer of Aed a 10.0101 (Q17H75).

We have also identified lysosomal aspartic protease (Aed a 4), an allergen with homologues in *A. fumigatus* (Asp f 10), *Blatella germanica* (Bla g 2), and *Periplaneta americana* (Per a 2); 26% of patients with allergic bronchopulmonary aspergillosis had specific IgE against Asp f 10.[30] In a cockroach-allergic population, Bla g 2 and Per a 2 showed frequency of IgE binding of 57.6%-63.4%.[33,34] The 40% of frequency of reactivity found in this study suggest that Aed a 4 is a minor allergen.

Aed a 8 is a member of the Heat-shock protein-70 (Hsp70) family, which are exploited by dengue virus[35] and mosquito-borne flaviviruses (encephalitis virus)[36] to attach and penetrate mosquito cells. Hsp70 allergens have been reported in cockroach[37] and *D. farinae*.[18] Aed a 8 reacted with the IgE of 60% of the tested sera and it could represent a major allergen. Similar frequency of reactivity was observed with Hsp70 from *D. farinae*.[18] Our results suggest that Aed a 8 is a pan-allergen which deserves further study.

Other potential pan-allergens identified in this study are the "arginine or creatine kinase" and GPDH. Arginine kinase in mites belongs to group 20 allergens[38] and in shrimp to group 2.[39] Arginine kinases from *Canis familiaris* (dog), *Bombyx mori* (Silk moth), *Crangon crangon* (North Sea shrimp), *P. americana* and *Plodia interpunctella* (Indian meal moth) are registered in the IUIS allergen database and detected in insects, shrimp, and wasps.[31,40,41] GPDH has been reported with IgE binding in insects, *Gryllus bimaculatus*,[40] banana shrimps, *Fenneropenaeus merguiensis*,[41] Asian wasps, and *Vespa affinis*.[31] We found that these proteins have an IgE binding ranging from 6.7% to 13.3%, suggesting that they are minor allergens.

One of the ultimate goals in allergy research is to implement a comprehensive panel of component-resolved diagnosis. This requires the identification and characterization of a complete set of allergens from the allergenic source and might be hampered by the presence of cross-reactive molecules which could drive to false-positive results. We have demonstrated that *A. aegypti* represents a source of several IgE-binding proteins. We suggest that a mixture of three allergens (Aed a 6, Aed a 8, and Aed a 10) may be enough to identify more than 80% of *A. aegypti*-allergic individuals. Although these findings may have a potential clinical implication, they must be confirmed in studies using purified natural or recombinant allergens in well-defined patient populations. Further biochemical, structural, and immunological studies are necessary to perform a comprehensive characterization of the complete set of allergens from *A. aegypti* and define their clinical relevance.

In a previous publication, we reported that the tropomyosin variant Aed a 10.0101 may contain the complete repertoire of IgE epitopes from the mosquito tropomyosin family.[11] The results from our study confirmed this observation, as rAed a 10.0101 produced complete inhibition of the IgE binding to the 32 kDa band (which represents the natural mosquito tropomyosins), while rAed a 10.0201 produced partial inhibition. Furthermore, this experiment demonstrated that the IgE binding of tropomyosin has no effect on the reactivity to the rest of the allergens, highlighting the relevance of these molecules. Interestingly, we found that specific IgE from patient 8 binds to Aed a 10.0201 but not to Aed a 10.0101 (Figure 2 and Table 3), suggesting that both tropomyosins should be considered.

In conclusion, we have identified and characterized a set of allergens from the mosquito species *A. aegypti*. Four proteins with homology to different allergen groups from other allergen sources were identified, which together with tropomyosin may partially explain the basis of the cross-reactivity between mosquito and other arthropods. Additionally, four proteins with inferred biological function and one without known biological function were also identified. Previous studies had focused on the saliva and salivary glands of *A. aegypti*, and four allergens have been reported as factors associated with the cutaneous reactions that appear after mosquito bites.[10,42-45] In this study, we did not detect these allergens as we used sera from individuals with respiratory allergy. We hypothesize that different allergenic components may be involved in the variable manifestations of mosquito allergy. Our results provide a basis for a more comprehensive understanding of the relationship between allergens in *A. aegypti* and in other arthropod species.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

JFC, LP, and EFC conceived and designed the experiments; JFC performed the experiments; JFC, LP, EFC, and PP wrote the paper and analyzed the data; SLM recruited and skin-tested the patients; LP, JLS, and EFC contributed reagents, materials, and analysis tools. All the authors revised and contributed to the manuscript.

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B. Production and purification of *A. aegypti* allergens and characterization of their molecular and immunological properties

In this part of the thesis, we purified native tropomyosin (Aed a 10) and expressed rAed a 8. Obtaining purified natural allergens is the most convenient way to study their role in allergies. In the case of mosquito allergens, this task is difficult given the low availability of pharmaceutical grade source materials (mainly whole mosquito bodies) and the problems associated to the purification protocols, which usually requires specialized laboratory infrastructure and is time consuming. Molecular cloning is one of the best options to circumvent these inconvenients. However, some allergens are highly represented in the extracts of other sources and the purification of the natural molecules is a good option.

Tropomyosin from *A. aegypti* is present in a high amount in the whole body extract. We applied a combination of chromatographic techniques for the purification of this allergen. The results revealed that this allergen corresponds to a mixture of at least four variants with IgE-binding capacity. Two variants seem to be most abundant. These allergens were obtained as recombinant molecules. Direct ELISA and ELISA inhibition experiments, and immunoblots allowed the characterization of these molecules. The two tropomyosins were accepted as allergens by the WHO/IUIS allergen nomenclature database with the names of Aed a 10.0101 and Aed a 10.0201.

The study of the Allergenome of *A. aegypti* revealed that Aed a 8 (Heat shock cognate-70) may represent a major allergen. The recombinant version of this allergen was expressed in *E. coli* and used to study its antibody binding properties. rAed a 8 was used to immunize mice and to study the immune response induced by this molecule. The results showed that rAed a 8 is immunogenic and has IgE and IgG-binding capacity. The response induced in mice was characterized by high levels of IgG2a, which indicates a possible immune-regulatory effect.

For further information see the following papers:

Article 3.

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Identification and Characterization of IgE-Binding Tropomyosins in *Aedes aegypti*

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Key Words
Aedes aegypti · IgE cross-reactivity · Mosquito allergy · Tropomyosin

Abstract

Background: The mosquito *Aedes aegypti* is a potential source of important clinically relevant allergens. However, the allergenicity and cross-reactivity of most of these has not been fully described. ***Methods:*** Natural wild-type mosquito tropomyosin was purified by size exclusion and anionic-exchange chromatography from an *A. aegypti* extract. Further characterization was accomplished by MALDI-TOF/TOF. Two recombinant variants of tropomyosin were obtained by expression in *Escherichia coli*. Specific IgE measurement by ELISA and skin tests for mosquito extract were performed in 12 patients with asthma or allergy rhinitis residing on the Caribbean island of Martinique. Cross-reactivity between natural *A. aegypti* tropomyosin and recombinant tropomyosins from *A. aegypti*, house dust mite, shrimp and *Ascaris lumbricoides* was analyzed by ELISA competition. ***Results:*** Four variants of natural tropomyosin were purified. A band of 32 kDa in SDS-PAGE representing 2 tropomyosin variants (Aed a 10.0101 and Aed a 10.0201) reacted with specific IgE of 4 of the 12 (33%) allergic patients and with rabbit polyclonal anti-shrimp tropomyosin. A high degree of cross-reactivity (60–70%) was detected between natural mosquito tropomyosin and Blo t 10, Der p 10 and Lit v 1, and a lower degree with Asc l 3 from *A. lumbricoides* (<30%). rAed a 10.0101 inhibited IgE binding to natural *A. aegypti* tropomyosin; however, rAed a 10.0201 showed a low inhibitory capacity. ***Conclusion:*** Tropomyosin is a new IgE-binding protein from *A. aegypti*. Two of the 4 variants identified showed different degree of cross-reactivity with tropomyosins from other arthropods. The potential allergenic role of each variant should be further investigated.

Introduction

Mosquitoes are insects that are distributed worldwide, and they cause IgE-mediated allergic responses in sensitized individuals [1]. Manifestations of mosquito allergy are variable and depend on the route of exposure. When the contact with the allergens occurs via skin bites (saliva allergens), the symptoms consist of mild or severe local or systemic reactions [1–4]. However, evidence suggests

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that the inhalation of mosquito emanations and detritus deposited in house dust may induce allergic respiratory symptoms such as allergic asthma and rhinitis [5]. This is supported by epidemiological studies which show that 2.5–47% of individuals with asthma and/or allergic rhinitis are sensitized to mosquito allergens [6–8].

We found that sera obtained from a cohort of patients residing on the Caribbean island of Martinique suffering from allergic respiratory symptoms after the inhalation of house dust mite allergens recognize several allergens from *Aedes aegypti* [9]. We have also demonstrated cross-reactivity between *A. aegypti* and house dust mites, shrimp and cockroach extracts [10]. These findings suggest that *A. aegypti* may contain allergens that participate in the pathogenesis of respiratory allergies as inducers of a Th2 response, and the subsequent development of allergic symptoms or as a modulator of the immune response established against mites, shrimp and/or cockroaches.

Tropomyosin is a pan-allergen that belongs to a family of phylogenetically conserved proteins with multiple isoforms present in both the muscle and nonmuscle cells of vertebrates and invertebrates [11]. Allergenic tropomyosins have been described in crustaceans, molluscs, arthropods, insects and helminths [12–17]. The participation of tropomyosin in cross-reactivity between mites, cockroaches and shrimps has been demonstrated. Despite the functional and structural similarity of tropomyosins, several of their presentations are nonallergenic [18]. In some regions of tropical countries where allergies to arthropods are common, most individuals are exposed concomitantly to other tropomyosin sources, such as those from helminths. This coexposure may have clinical implications, either by modulating [19, 20] or exacerbating [21–23] a previously mounted allergic response. In these regions, *A. aegypti* and other mosquito species are highly prevalent.

The aim of this study was to purify natural wild-type *A. aegypti* tropomyosin variants and to characterize the IgE-binding capacity of these molecules. As further characterization, we cloned and expressed 2 full-length recombinant tropomyosin variants and compared their antibody-binding capacity with natural tropomyosin.

Methods

Sera

Sera were obtained from 24 (14 males and 10 females) allergic patients who attended an outpatient Allergy Clinic in Fort de France, Martinique. All patients were skin tested with a panel of common standardized inhalant allergens and with a commercially available *Aedes communis* extract (Stallergènes, Antony, France). Patients who developed a wheal diameter >3 mm to the extracts were considered to have a positive skin prick test. Twelve of these patients had a test that was positive to mosquito extract and other arthropods (table 1). Sera were obtained after written consent was signed in the context of the study, entitled: 'Etude Epidémiologique des causes Allergiques de l'asthme en Martinique', which was funded and approved by 'Fonds de'aide à la qualite de soins de ville' from 'la Caisse Generale de Securite Sociale de la Martinique' and the 'Comite Departemental de la Martinique contre les maladies respiratoires et la tuberculose (CDMMRT)'.

Preparation of A. aegypti Extract

Lyophilized and defatted *A. aegypti* raw material was purchased from GREER Labs (Lenoir, N.C., USA) and extracted 1:40 overnight (ON) with 0.1 M PBS, pH 7.2 at 4°C with constant stirring. After extraction, the mixture was centrifuged at 10,000 rpm over 10 min, the supernatant was filtered through a 0.2-μm membrane, dialyzed against distilled water in a 3,500-Da cut-off membrane (Spectra/Por Dialysis Membranes, Houston, Tex., USA) and lyophilized until usage. The protein concentration was determined by Bradford assay.

Shrimp, Mite and Nematode Recombinant Tropomyosins

Production of recombinant (r) tropomyosin from the white shrimp *Litopenaeus vannamei* (rLit v 1) was performed as follows: the nucleotide sequence of tropomyosin of *L. vannamei* (GenBank EU410072.1) was optimized for codon utilization in an *Escherichia coli* expression system, obtained by gene synthesis and cloned into the expression vector pET45b+ by GenScript (Piscataway, N.J. USA). A single colony of transformed *E. coli* BL21 was grown overnight at 37°C in LB medium with ampicillin (LBA). Afterwards, the culture was diluted 1:20 in LBA and grown at 37°C to an OD_{600} of 0.5 nm. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. A cell pellet was collected by centrifugation and resuspended in denaturating binding buffer (pH 7.8) and then sonicated. His-tagged protein was purified on an Ni-NTA column under hybrid conditions (ProBond Purification System, Invitrogen), lyophilized and stored at –20°C. Production of recombinant tropomyosins from the mites *Dermatophagoides pteronyssinus* (rDer p10), *Blomia tropicalis* (rBlo t 10) and *A. lumbricoides* (rAsc l 3) was performed as previously described [17, 24].

Purification of Natural Wild-Type Tropomyosin from A. aegypti

Purification was performed in 2 sequential steps consisting of gel filtration and ionic-exchange high-performance liquid chromatography. In the first step, the lyophilized whole-body mosquito extract was reconstituted in 2 ml of 0.1 M PBS to a final concentration of 6 mg/ml, filtered through a 0.2-μm membrane and prepurified by gel filtration in a HiLoad Superdex 75 prep grade, 16/600-cm column (GE Healthcare Life Sciences, Amersham, UK). The molecule of interest was eluted by applying 0.1 M PBS at a flow of 1 ml/min, and the detection was performed at a wavelength of 280 nm. Several fractions were collected and analyzed by SDS-PAGE under reducing conditions. Fractions containing the band of 32 KDa were dialyzed in a 3,500-Da cut-off membrane (Spectra/Por) against water and lyophilized for further purification by anion-exchange chromatography. The mobile phases were

Table 1. Clinical details and sensitization profiles of 12 allergic individuals used in the study

Serum	Age, years	Gender	Diagnosis	Mean wheal diameter, mm						Total IgE, IU/ml	Specific IgE against			
				A. communis	*D. pteronyssinus*	*D. farinae*	*B. tropicalis*	shrimp	cockroach		*A. aegypti* extract	native tropomyosin	rAed a 10.0101	rAed a 10.0201
1	3	M	A	6	10	6	9.5	4	0	1,260	0.379	0.052	0.062	0.077
2	8	M	R, C	4	0	0	0	0	0	626.4	0.165	0.047	0.063	0.069
3	4	M	A, C	3.5	5	3.5	4	0	0	2,187	0.458	0.386[a]	0.289[a]	0.158[a]
4	9	M	A, C	4.5	5	4.5	8	3.5	3	630	0.173	0.075	0.077	0.089
5	11	M	A, R, C	4	5.5	6	5	5	3.5	2,095.4	1.679	1.683[a]	1.417[a]	0.991[a]
6	5	M	A, C	3	9.5	7	3.5	0	3	324	0.319	0.052	0.078	0.076
7	13	M	R, C	6	4	3.5	3.5	0	3	2,832	0.186	0.047	0.065	0.070
8	27	F	R	5	0	0	0	0	6.5	545.5	0.355	0.179[a]	0.480[a]	0.182[a]
9	15	M	A, R, C	>3	>3	>3	>3	>3	>3	1,246	1.239	0.582[a]	0.403[a]	0.300[a]
10	9	M	R, C	>3	0	0	0	0	0	611	0.111	0.059	0.064	0.067
11	5	M	R, C	>3	>3	>3	>3	>3	>3	761	0.275	0.061	0.068	0.077
12	37	F	R, C	>3	0	0	0	0	0	58	0.332	0.051	0.070	0.063

A = Asthma; C = conjunctivitis; F = female; M = male; R = rhinitis.

[a] Positive specific IgE.

phase A: 10 mM sodium phosphate buffer (pH 6.0) and phase B: 10 mM sodium phosphate buffer (pH 6.0) with 1.0 M NaCl. The lyophilized fraction was reconstituted in phase A and injected onto a HiPrep QFF 16/10 cm column (GE Healthcare Life Sciences). The removal of unbound molecules was carried out in a cleaning step with phase A. Bound proteins were eluted through a binary gradient of phase A and B at a flow of 3 ml/min, and the detection was performed at a wavelength of 280 nm. Fractions corresponding to the peaks that appear between 0.30 and 0.45 M NaCl were dialyzed against water and lyophilized for further analysis.

Passive Elution of Tropomyosin from Electrophoresed Gels

The fraction obtained from the HiPreP QFF column showed 4 main bands, with the 32-kDa band being the most intense. To further purify this band, the sample was separated by SDS-PAGE under reducing conditions and the band was excised from the stained gel with a clean razor. Gel pieces were placed in microcentrifuge tubes, immersed in elution buffer (50 mM Tris-HCl, 150 mM NaCl and 0.1 mM EDTA, pH 7.5), crushed using a clean pestle and then incubated in a rotary shaker at 37°C ON. The eluted protein was recovered by centrifugation at 10,000 rpm and filtration through a 0.45-μm membrane. Eluted protein was further characterized by ELISA, ELISA competition and mass spectrometry (MS).

SDS-PAGE and Immunoblotting

The mosquito extract and purified fractions were separated by SDS-PAGE under denaturing conditions using a Mini-PROTEAN II apparatus (Bio-Rad, Hercules, Calif., USA). Ten micrograms of extract or 2 μg of the purified proteins per slot were separated in 12% polyacrylamide gels at 120 volts, and the gels were stained with GelCode Blue stain reagent (Life Technologies, Carlsbad, Calif., USA). For immunoblot, proteins were electrotransferred from the polyacrilamide gel onto 0.45-μm nitrocellulose membranes using a Trans-Blot Semi-Dry electrophoresis transfer cell (Bio-Rad), at 20 v, and blocked with PBS (pH 7.4) and 0.25% Tween 20 (PBS-T), containing 5% BSA. Membranes were washed twice with PBS-T and incubated with pooled sera (sera 3, 5, 8 and 9; table 1) from allergic individuals, diluted 1:10 in PBS-T containing 1% BSA for 2 h at 4°C. After washing with PBS-T, the membranes were incubated with 1:2,000 diluted horseradish peroxidase (HRP)-conjugated mouse anti-human IgE Fc (SouthernBiotech Birmingham, Ala., USA) for 1 h at 4°C, washed again, and then incubated with ECL prime detection reagent (Ge Healthcare Life Sciences). Chemiluminescence was detected using GeneGnome apparatus (Syngene, Cambridge, UK). Otherwise, for the detection of tropomyosin, the electrotransferred and blocked membranes were incubated with 1:5,000 diluted polyclonal antibodies obtained from rabbits immunized with shrimp tropomyosin (Pen m 1; Bial Aristegui, Lisbon, Portugal), that can also bind to tropomyosins from other sources [12, 25, 26], and 1:2,000 diluted HRP-conjugated anti-rabbit IgG (Sigma-Aldrich, St. Louis, Mo., USA). Detection of bands was performed as above.

For inhibition experiments with shrimp tropomyosin, nitrocellulose strips with transferred proteins were incubated with 2 ml of pooled sera diluted 1:4 (sera 3, 5, 8 and 9; table 1) previously adsorbed ON with 10 μg/ml of Lit v 1. IgE was detected as described before.

Expression and Purification of Recombinant A. aegypti Tropomyosins

Production and purification of 2 tropomyosin variants was achieved as follows. The nucleotide sequence of 2 tropomyosin variants (GenBank EAT46014.1 for Aed a 10.0101 and EAT46008.1 for Aed a 10.0201) were synthesized at GenScript (Township, N.J., USA). The nucleotide sequence of Aed a 10.0201 was modified by codon optimization based on the OptimumGene™ algorithm. The synthesized nucleotide sequence of Aed a 10.0101 was subcloned into pCold IV vector (Clontech, Otsu, Japan) and the nucleotide sequence of Aed a 10.0201 was subcloned into pET-14b vector (Novagen, Billerica, Mass., USA). The vectors were used to transform *E. coli* One Shot BL21(DE3) (Life Technologies). Transformed *E. coli* was grown at 37°C in LBA with shaking at 250 rpm, up to an OD_{600} of 0.5 nm. The induction of tropomyosin expression in cells harboring pET-14b/Aed a 10.0201 plasmid was achieved by the addition of 0.5 mM IPTG and incubation for 5 h. Cells harboring pCold IV/Aed a 10.0101 plasmid were subjected to thermal shock at 15°C and incubated at this temperature during 24 h in the presence of 0.5 mM IPTG. Afterwards, the cells were harvested by centrifugation at 10,000 rpm for 10 min. Tropomyosin was partially purified by isoelectric precipitation. The cell pellet was lysed in 20 mM Tris-HCl, 1.0 M KCl, 10 mM β-mercaptoethanol (pH 7.5) and boiled at 97°C for 5 min and then centrifuged at 10,000 rpm for 5 min. The pH of the supernatant was adjusted at 4.85 with 1.0 M HCl for isoelectric precipitation, and stirred for 1 h at 4°C. The sample was centrifuged again and the precipitate dissolved in 20 mM of phosphate buffer (pH 7.4) and 0.5 M NaCl.

Purification of rAed a 10.0101 was performed following the strategy proposed by Erban [27], with some modifications. The sample containing the recombinant tropomyosin was subjected to ammonium sulfate fractionation. The protein extracted at 45% of ammonium sulfate was dialyzed against Tris-buffered saline (50 mM Tris- HCl, pH 7.4, and 150 mM NaCl) and the sample loaded onto a HiTrap Benzamidine FF (high sub) column (GE Healthcare Life Sciences) and then washed with binding buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4). Finally, 0.05 M glycine (pH 3.0) was used to elute purified protein. Fractions containing recombinant tropomyosin were pooled, dialyzed in 3,500-Da cut-off membrane (Spectra/Por) and lyophilized until usage.

Purification of rAed a 10.0201 was performed in Ni-NTA resin (Life Technologies) under native conditions, according to the manufacturer's instructions. Protein fractions were analyzed by SDS-PAGE and those containing recombinant tropomyosin were pooled, dialyzed as above and lyophilized until usage.

Enzyme-Linked Immunosorbent Assay

For specific-IgE level measurement, microplates were coated ON with 0.5 μg of extract or 0.05 μg of purified tropomyosin per well, and diluted in 100 μl of 0.05 M carbonate/bicarbonate buffer (pH 9.6). After washing 3 times with PBS-T, nonspecific binding was blocked for 1 h with PBS-T containing 1% BSA, followed by 3 more washing steps. Microplates were then incubated for 2 h with sera, diluted 1:8 in PBS-T containing 1% BSA and, after washing, incubated for 1 h with 1:2,000 diluted HRP-conjugated mouse anti-human IgE Fc (Southern Biotech). Finally, microplates were washed and incubated with *o*-phenylenediamine dihydrochloride (Sigma-Aldrich). Reaction was stopped with HCl and absorbance was read at 492 nm. All samples were assayed in duplicate. Sera from 9 subjects with pollen allergy were used as negative controls. 'No antigen' background values were subtracted from test sera data. Three standard deviations above the mean OD_{492} nm value from the control subject sera were used to determine the cut-off for positive IgE binding.

ELISA Competition Assay

To study the cross-reactivity between natural *A. aegypti* tropomyosin and tropomyosin from other organisms (rDer p 10, rBlo t 10, rLit v 1 and rAsc l 3) or between natural *A. aegypti* tropomyosin and the 2 recombinant tropomyosin variants, ELISA competition assays were performed. *Betula verrucosa* extract prepared at Inmunotek, SL, Madrid, Spain, was used as negative control. A pool of sera from 3 allergic patients (sera 3, 5 and 9; table 1) with IgE against purified natural *A. aegypti* tropomyosin were diluted 1:4 in PBS-T containing 1% BSA. Microplates were coated with antigen and blocked as described above. Subsequently, 50 μl of serially diluted competitor and 50 μl of diluted sera were added to the wells. The final dilution of the sera was 1:8. Microplates were incubated ON at 4°C. The reaction was developed as described before. The pool adsorbed with PBS-T containing 1% BSA without competitor served as a positive control. Percent inhibition was calculated using the following equation: percent inhibition = 100 – [(OD of serum with competitor/OD of serum without competitor) × 100].

Proteomic Analysis

MS analysis was realized at the Hospital Nacional de Parapléjicos Proteomic Unit, Toledo, Spain, as previously described [28]. The band of 32 kDa was excised from the 1-dimensional electrophoresis gel, reduced with 10 mM dithiothreitol (Sigma-Aldrich), alkylated with 55 mM iodoacetamide (Sigma-Aldrich) and digested with sequencing-grade modified porcine trypsin (Promega, Madison, Wis., USA) at a final concentration of 12.5 ng/μl according to Shevchenko et al. [29]. Peptides were desalted on a precolumn (Acclaim PepMap 100 C18, 5 μm, 100Å; 300 μm i.d. × 5 mm, LC Packings), followed by separation on an UltiMate™ Nano LC system (Dionex) using an Onyx Monolithic C18 column (150 × 0.1 mm, Phenomenex Inc., Torrance, Calif., USA). Fractionation of the peptides was performed with a Probot™ Microfraction Collector (Dionex). CHCA (Sigma-Aldrich) was used as a MALDI matrix. MS and tandem MS (MS/MS) analysis of offline spotted peptide samples were performed using the 4800 plus MALDI TOF/TOF Analyzer mass spectrometer (Sciex). The parent ion of Glu-1 fibrinopeptide (Sigma-Aldrich) at m/z 1570.677, diluted in the matrix (2 fmol per spot), was used for internal calibration. Up to 10 of the most intense ion signals per spot position, with s/n >40 were selected as precursor for MS/MS acquisition, excluding common trypsin autolysis peaks and matrix ion signal. Peptide and protein identifications were performed using ProteinPilot™ software v5.0 (Sciex) and the Paragon algorithm [30]. Each MS/MS spectrum was searched against a Uniprot/Swiss-Prot protein sequence database entry (October 2013), with the fixed modification of carbamidomethyl-labeled cysteine parameter enabled. Other parameters, such as the tryptic cleavage specificity, the precursor ion mass accuracy and the fragment ion mass accuracy, are MALDI 4800 built-in functions of ProteinPilot software. A search against a concatenated database containing both forward and reversed sequences enabled the false discovery rate to be kept at <1%.

Results

Purification of Tropomyosin from an A. aegypti Extract

The fraction collected at retention times of 40–52 min in the gel filtration contained a 32-kDa band, which bound specific IgE. When separated by ionic-exchange chromatography, this band showed a spectrum with peaks II and III at 0.30–0.45 M NaCl containing proteins of interest (fig. 1a). The immunoblotting showed that peak II corresponded to a mixture of 4 principal bands of 50, 32, 27 and 18 kDa, which also reacted with a human

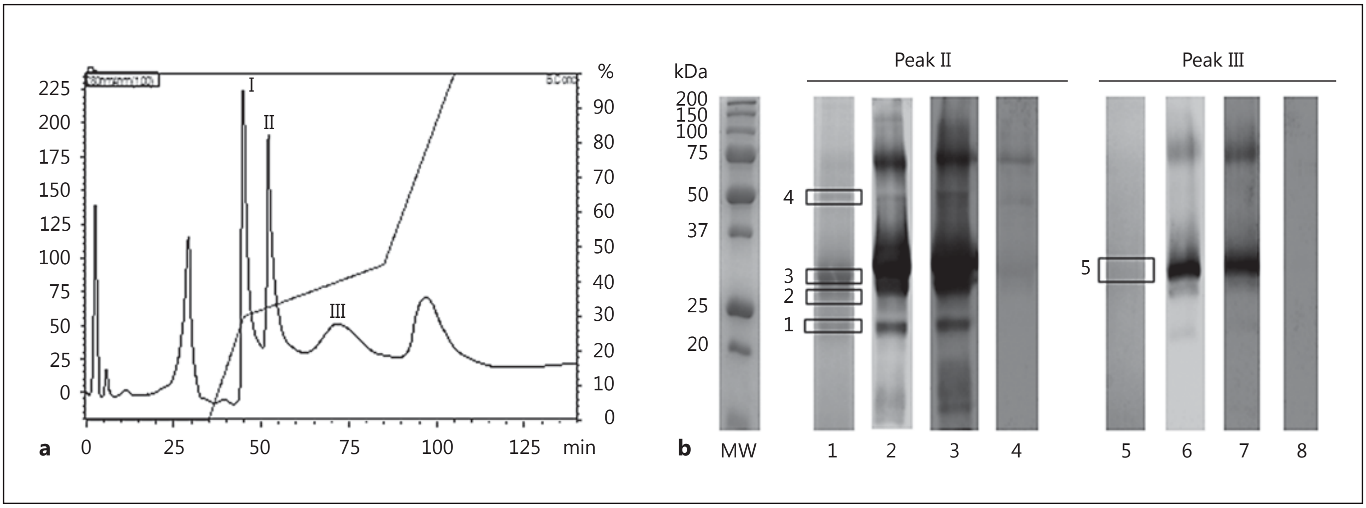

Fig. 1. Purification and IgE reactivity of natural tropomyosin from *A. aegypti*. **a** Ionic-exchange chromatography spectrum. **b** Peaks II and III obtained by ionic exchange contained 5 principal bands in the SDS-PAGE (strips 1 and 5) and reacted with serum IgE from allergic patients (strips 2 and 6) and polyclonal rabbit anti-shrimp tropomyosin (strips 3 and 7). Adsorption of human pooled sera with 10 μg/ml rLit v 1 abrogated the reactivity (strips 4 and 8).

Table 2. Proteins obtained from an *A. aegypti* extract after purification by gel electrophoresis and ionic-exchange liquid chromatography and identified by MS/MS analysis

Band No.	Protein	Uniprot code	Coverage, %	Theoretical MW, kDa	Experimental MW, kDa
1	myosin	Q178Y4	19.87	224.21	18
2	tropomyosin	Q17H76	76.75	31.001	27
3	tropomyosin	Q17H75	78.59	32.722	32
		Q17H80	78.87	32.465	32
4	myosin	Q178Y4	27.05	224.21	50
5	tropomyosin	Q17H76	52.76	31.001	32
		Q17H82	47.53	32.376	32

serum with positive IgE to *A. aegypti* and the rabbit anti-Pen m 1 polyclonal antibody (fig. 1b). The reactivity significantly decreased when both human and rabbit sera had been incubated with 10 μg of Lit v 1, suggesting that the bands contained tropomyosin, which cross-react with shrimp tropomyosin.

Characterization of Natural Tropomyosins from Purified Fractions

MS/MS analysis enabled the identification of 4 tropomyosin versions, previously reported in Uniprot (Uniprot codes Q17H75, Q17H76, Q17H80 and Q17H82) and 2 myosin products (table 2). The 32-kDa band excised from the SDS-PAGE gel corresponded to a mixture of 2 tropomyosins (Q17H75 and Q17H80) which showed IgE binding capacity. Even though these 2 molecules migrated at the same level in the gel, our MS/MS analysis was accurate enough to determine the sequence of specific peptides which enabled us to identify the presence of these 2 protein variants. Amino acid sequence alignment analysis showed 95.1% identity between Q17H75 and Q17H76, and 96.1% identity between Q17H80 and Q17H82. However, the percentage of identity between Q17H75 and Q17H80, and between Q17H82 and Q17H76, was 63% and 63.5%, respectively.

The band 3 (the mixture of Q17H75 and Q17H80 variants) showed IgE reactivity with 4 of 12 sera (33.3%) (table 1). The IUIS Allergen Nomenclature Subcommittee

Q17H75 (Aed a 10.0101)

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        10         20         30         40         50
MDAIKKKMQA MKLEKDNALD RALLCEQQAR DANLRAEKAE EEARQLQKKI
        60         70         80         90        100
QAIENDLDQT QEALMAVNAK LEEKEKALQN AESEVAALNR RIQLLEEDLE
       110        120        130        140        150
RSEERLASAT AKLSEASAAA DESERARKVL ENRALADEER MDALENQLKE
       160        170        180        190        200
ARFMAEEADK KYDEVARKLA MVEADLERAE ERAEAGEGKI VELEEELRVV
       210        220        230        240        250
GNNLKSLEVS EEKANQREEE YKNQIKTLTT RLKEAEARAE FAERSVQKLQ
       260        270        280
KEVDRLEDEL VMEKEKYREI GDDLDTAFVE LILKE
```

Q17H80 (Aed a 10.0201)

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        10         20         30         40         50
MDAIKKKMQA MKLEKDNAAD KADTCENQAK EANLRADKIM EEVAELTKRL
        60         70         80         90        100
TQVTEDHEKF KNTLEQANKD LEEKEKLLTS TEANVAALTR KVQQVEEDLE
       110        120        130        140        150
KSEERSGAAL SKLLEATQSA DENNRMCKVL ENRSQQDEER MDQLSNQLKE
       160        170        180        190        200
ARMLAEDADG KSDEVSRKLA FVEDELEVAE DRVKSGEAKI MELEEELKVV
       210        220        230        240        250
GNSLKSLEVS EDKANQRVEE FKRQLKSLTI KLKEAETRAE NAEKNVKKLQ
       260        270        280
KEVDRLEDKL MNEKDKYKAI CDDLDSTFAE LTGY
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Fig. 2. Amino acid sequences of 2 *A. aegypti* tropomyosins (Aed a 10.0101 and Aed a 10.0201). A sample from ionic-exchange chromatography (fig. 1b: band 3, strip 1) was analyzed by MS/MS. The peptide sequences identified are highlighted.

(www.allergen.org) has assigned to these tropomyosins the following official numbers: Aed a 10.0101 for Q17H75 and Aed a 10.0201 for Q17H80. These allergens share 60.2% of identity in the amino acid sequence (fig. 2).

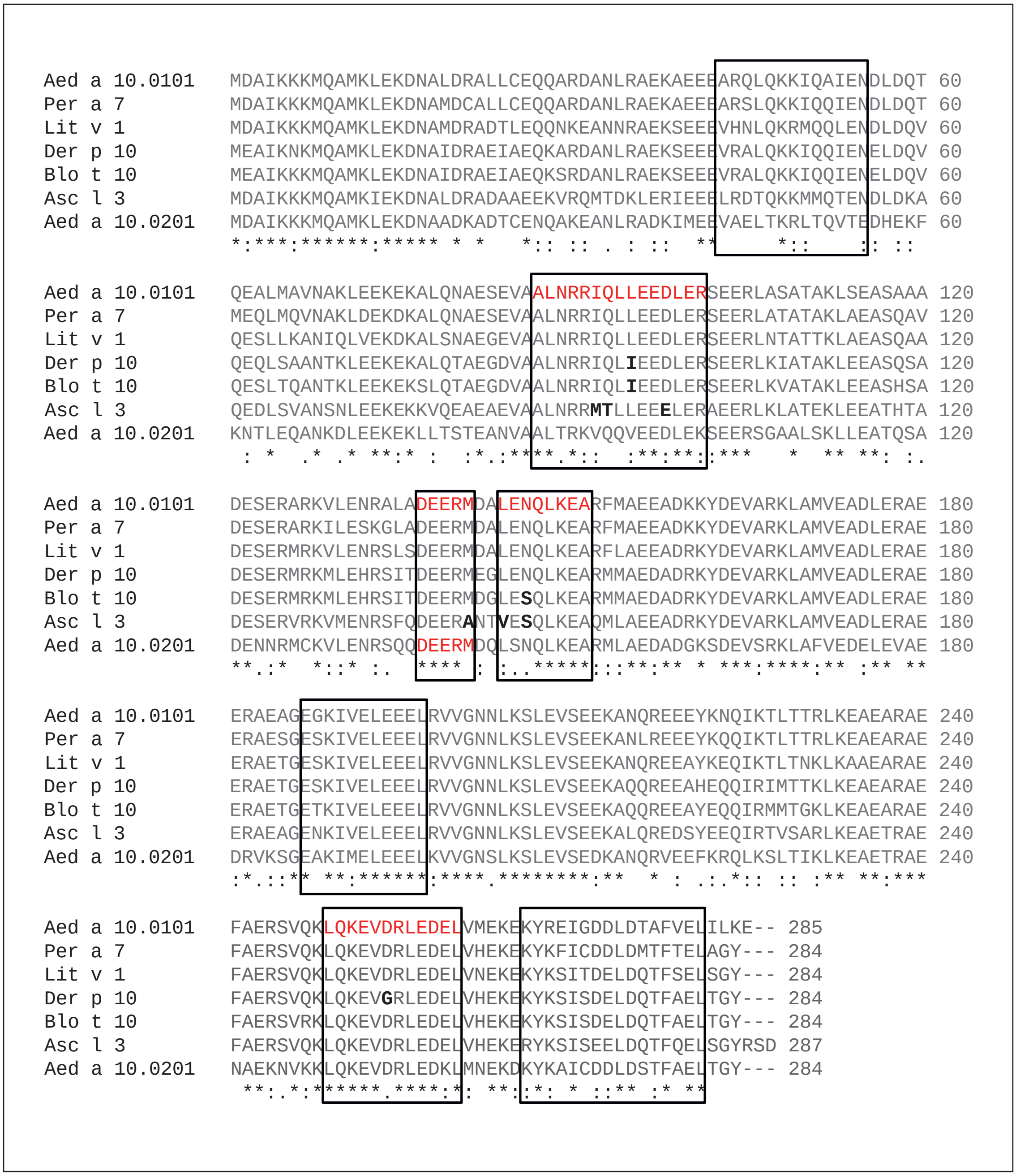
Analysis of sequence alignments showed a greater similarity of Aed a 10.0101 with the other arthropod tropomyosins than that shown by Aed a 10.0201. The comparative analysis of amino acid sequence of the IgE-binding epitopes of tropomyosin from mite, cockroach and shrimp [31–33] also showed the greater similarity of the Aed a 10.0101 variant in these epitope regions (fig. 3). Thus, 4 putative epitopes, corresponding to residues 87–101 (ALNRRIQLLEEDLER), 137–141 (DEERM), 144–151 (LENQLKEA) and 249–260 (LQKEVDRLEDEL), were completely conserved in the amino acid sequence of Aed a 10.0101. The epitope ESKIVELEEEL (residues 187–197) is modified by a single change EGKIVELEEEL in Aed a 10.0101. Only 1 epitope, corresponding to residues 137–141, was conserved in Aed a 10.0201(fig. 3).

Tropomyosin from A. aegypti Cross-Reacts with the Other Allergenic Tropomyosins

ELISA competition assays showed that when *A. aegypti* tropomyosin was in the solid phase, homologous inhibition was 61.48% at 2 µg/ml of competitor. At this concentration, rBlo t 10, rDer p 10 and rLit v1 reached 75, 73.98 and 61.54% of inhibition respectively, whereas rAsc l 3 reached only 23.68% (fig. 4).

Recombinant Aed a 10.0101 and Aed a 10.0201 Tropomyosins Showed Different IgE-Binding Capacities

On SDS-PAGE, rAed a 10.0101 migrated with an apparent molecular weight (MW) of 32 kDa and rAed a 10.0201 migrated at 34 kDa. Both molecules exhibited IgE- and IgG-binding capacities when incubated with human sera and rabbit anti-shrimp tropomyosin antibody, respectively (fig. 5). However, bands with a higher MW that may correspond to dimers or trimers were also observed in the immunoblotting. Cross-inhibition by IgE competition ELISA between the natural purified tropomyosin mixture and both recombinant variants showed that Aed a 10.0101, or an equimolar mixture of Aed a 10.0101 and Aed a 10.0201, were able to abrogate all the IgE binding to the natural tropomyosin mixture. In contrast, competition with Aed a 10.0201 only inhibited the IgE reactivity to natural tropomyosin up to 30% (fig. 6).



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Aed a 10.0101    MDAIKKKMQAMKLEKDNALDRALLCEQQARDANLRAEKAEEEARQLQKKIQAIENDLDQT 60
Per a 7          MDAIKKKMQAMKLEKDNAMDCALLCEQQARDANLRAEKAEEEARSLQKKIQQIENDLDQT 60
Lit v 1          MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV 60
Der p 10         MEAIKNKMQAMKLEKDNAIDRAEIAEQKARDANLRAEKSEEEVRALQKKIQQIENELDQV 60
Blo t 10         MEAIKKKMQAMKLEKDNAIDRAEIAEQKSRDANLRAEKSEEEVRALQKKIQQIENELDQV 60
Asc l 3          MDAIKKKMQAMKIEKDNALDRADAAEEKVRQMTDKLERIEEELRDTQKKMMQTENDLDKA 60
Aed a 10.0201    MDAIKKKMQAMKLEKDNAADKADTCENQAKEANLRADKIMEEVAELTKRLTQVTEDHEKF 60
                 *:***:******:***** * *   *:: :: . : ::  **     *::    :: ::

Aed a 10.0101    QEALMAVNAKLEEKEKALQNAESEVAALNRRIQLLEEDLERSEERLASATAKLSEASAAA 120
Per a 7          MEQLMQVNAKLDEKDKALQNAESEVAALNRRIQLLEEDLERSEERLATATAKLAEASQAV 120
Lit v 1          QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAEASQAA 120
Der p 10         QEQLSAANTKLEEKEKALQTAEGDVAALNRRIQLIEEDLERSEERLKIATAKLEEASQSA 120
Blo t 10         QESLTQANTKLEEKEKSLQTAEGDVAALNRRIQLIEEDLERSEERLKVATAKLEEASHSA 120
Asc l 3          QEDLSVANSNLEEKEKKVQEAEAEVAALNRRMTLLEEELERAEERLKLATEKLEEATHTA 120
Aed a 10.0201    KNTLEQANKDLEEKEKLLTSTEANVAALTRKVQQVEEDLEKSEERSGAALSKLLEATQSA 120
                  : *  .* .* **:* :  :*.:****.*::  :**:**::***   *  ** **: :.

Aed a 10.0101    DESERARKVLENRALADEERMDALENQLKEARFMAEEADKKYDEVARKLAMVEADLERAE 180
Per a 7          DESERARKILESKGLADEERMDALENQLKEARFMAEEADKKYDEVARKLAMVEADLERAE 180
Lit v 1          DESERMRKVLENRSLSDEERMDALENQLKEARFLAEEADRKYDEVARKLAMVEADLERAE 180
Der p 10         DESERMRKMLEHRSITDEERMEGLENQLKEARMMAEDADRKYDEVARKLAMVEADLERAE 180
Blo t 10         DESERMRKMLEHRSITDEERMDGLESQLKEARMMAEDADRKYDEVARKLAMVEADLERAE 180
Asc l 3          DESERVRKVMENRSFQDEERANTVESQLKEAQMLAEEADRKYDEVARKLAMVEADLERAE 180
Aed a 10.0201    DENNRMCKVLENRSQQDEERMDQLSNQLKEARMLAEDADGKSDEVSRKLAFVEDELEVAE 180
                 **.:*  *::* :.  **** : :..*****:::**:** * ***:****:** :** **

Aed a 10.0101    ERAEAGEGKIVELEEELRVVGNNLKSLEVSEEKANQREEEYKNQIKTLTTRLKEAEARAE 240
Per a 7          ERAESGESKIVELEEELRVVGNNLKSLEVSEEKANLREEEYKQQIKTLTTRLKEAEARAE 240
Lit v 1          ERAETGESKIVELEEELRVVGNNLKSLEVSEEKANQREEAYKEQIKTLTNKLKAAEARAE 240
Der p 10         ERAETGESKIVELEEELRVVGNNLKSLEVSEEKAQQREEAHEQQIRIMTTKLKEAEARAE 240
Blo t 10         ERAETGETKIVELEEELRVVGNNLKSLEVSEEKAQQREEAYEQQIRMMTGKLKEAEARAE 240
Asc l 3          ERAEAGENKIVELEEELRVVGNNLKSLEVSEEKALQREDSYEEQIRTVSARLKEAETRAE 240
Aed a 10.0201    DRVKSGEAKIMELEEELKVVGNSLKSLEVSEDKANQRVEEFKRQLKSLTIKLKEAETRAE 240
                 :*.::** **:******:****.********:**  * : .:.*:: :: :** **:***

Aed a 10.0101    FAERSVQKLQKEVDRLEDELVMEKEKYREIGDDLDTAFVELILKE-- 285
Per a 7          FAERSVQKLQKEVDRLEDELVHEKEKYKFICDDLDMTFTELAGY--- 284
Lit v 1          FAERSVQKLQKEVDRLEDELVNEKEKYKSITDELDQTFSELSGY--- 284
Der p 10         FAERSVQKLQKEVGRLEDELVHEKEKYKSISDELDQTFAELTGY--- 284
Blo t 10         FAERSVRKLQKEVDRLEDELVHEKEKYKSISDELDQTFAELTGY--- 284
Asc l 3          FAERSVQKLQKEVDRLEDELVHEKERYKSISEELDQTFQELSGYRSD 287
Aed a 10.0201    NAEKNVKKLQKEVDRLEDKLMNEKDKYKAICDDLDSTFAELTGY--- 284
                  **:.*:******.****:*: **::*: * ::** :* **
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Color version available online

Fig. 3. Multiple-sequence alignment between *A. aegypti* tropomyosins (Aed a 10.0101 and Aed a 10.0201) and other tropomyosins. IgE-binding epitopes of Pen m 1 are indicated in boxes (described by Ayuso et al. [32]). Conserved epitopes in Aed a 10.0101 and Aed a 10.0201 are highlighted in red. Differences in the amino acid sequences of Der p 10, Blo t 10 and Asc l 3 are indicated in bold type.

Discussion

In this work, we report that tropomyosin from *A. aegypti* has IgE reactivity and its natural source is represented for at least 4 variants, 2 of them more related to tropomyosin from mites and shrimp than to that from *Ascaris lumbricoides*. Although >3,500 species of mosquito have been described, we have focused our work on *A. aegypti* because this mosquito has experienced a rapid global spread and is commonly found in America, central and southern Africa, Oceania, Asia and, to a lesser extent, in Europe [34], where it acts as a vector of several pathogens [35, 36]. Furthermore, *A. aegypti* has evolved to cohabit and feed from humans [37] who may be constantly exposed to the mosquito antigens.

Using a combination of 2 chromatographic techniques and proteomic analysis, 4 tropomyosin variants were identified in this study. These variants coeluted with truncated myosin on the ion-exchange column, which can be explained by the common association of

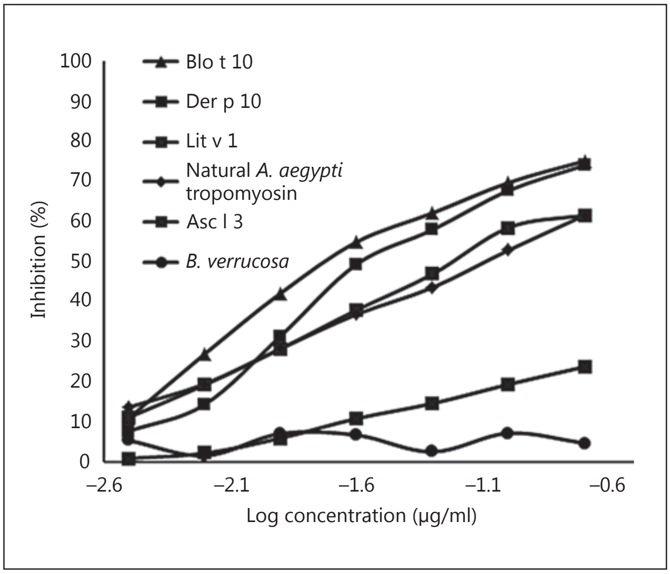

Fig. 4. IgE competition analysis. Microplate wells coated with natural *A. aegypti* tropomyosin were incubated with human pooled sera and different concentrations of rBlo t 10, rDer p 10, rLit v 1, rAsc l 3, natural *A. aegypti* tropomyosin or *B. verrucosa* extract.

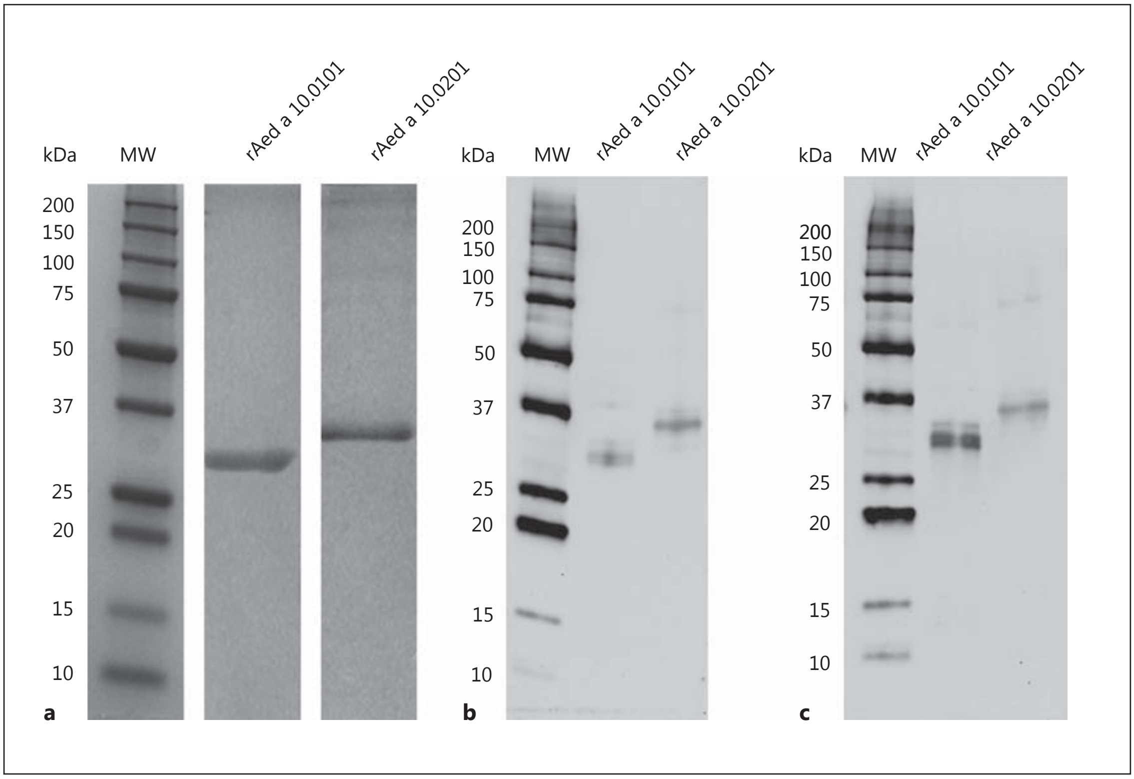

Fig. 5. a Gel electrophoresis and Coomassie staining of rAed a 10.0101 and rAed a 10.0201. Immunoblots of these recombinants incubated with pooled sera from allergic patients (**b**) or rabbit polyclonal anti-shrimp tropomyosin (**c**).

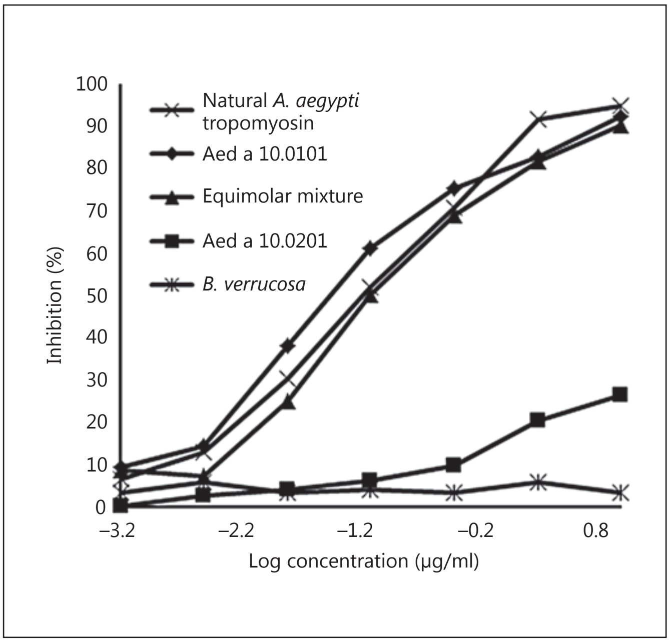

Fig. 6. IgE competition ELISA results. Microplate wells coated with natural *A. aegypti* tropomyosin were incubated with human pooled sera and different concentrations of rAed a 10.0101, rAed a 10.0210 or an equimolar mixture of these recombinants. Natural *A. aegypti* tropomyosin for homologous inhibition and *B. verrucosa* extract as unrelated antigens were used. At the highest concentration of the competitor rAed a 10.0201, 30% IgE-binding inhibition was obtained.

these proteins to the thin filament and microfilaments, together with actin, in all eukaryotic cells [11]. A band of 32 kDa was identified as a mixture of 2 variants, Aed a 10.0101 and Aed a 10.0201. These molecules have similar physicochemical characteristics, inducing them to eluting at the same retention time in gel-filtration and ion-exchange chromatographic techniques. The mixture of the natural tropomyosins reacted with the IgE in 33% of the allergic individuals. Further experiments should be performed in order to clarify the contribution of each variant in the pattern of reactivity observed. The higher degree of cross-reactivity between the natural mosquito tropomyosin and tropomyosin from mites and shrimp is consistent with the amino acid sequence analysis, suggesting the presence of potential IgE cross-reacting epitopes as well as with the close taxonomic relationship across these species.

The cross-reactivity study allowed us to hypothesize that mite tropomyosin is probably the primary source of sensitization, which means that sensitization to mosquito tropomyosins is influenced by sensitization to mite allergens. This aspect may have important clinical implications, i.e. the diagnosis of arthropod-induced allergy may be biased by cosensitization to *A. aegypti*, and this mosquito species may represent a source of IgE-cross-reactive molecules and not be a genuine sensitizing molecule. On the other hand, it is unknown if exposure to *A. aegypti* tropomyosin could have a role in the regulation of immune response induced by mite and shrimp tropomyosins.

In Africa and South America, the prevalence of sensitization to mite tropomyosin is approximately 50% [38, 39], higher than those observed in developed countries [40, 41]. These differences are partially explained by the presence of other Th2-inducing and cross-reactive antigens, such as *Ascaris* [17, 23]. Our results of specific IgE-binding and cross-reactivity suggest that *A. aegypti* tropomyosin may play a role in the sensitization pattern of allergic populations residing in areas where mosquitoes are highly prevalent.

The alignment of amino acid sequences of Aed a 10.0101 and Aed a 10.0201 with those from other arthropods suggests the presence of putative IgE epitopes, which are not yet experimentally verified. Aed a 10.0101 showed a higher sequence identity with tropomyosins from other allergenic sources when compared to Aed a 10.0201 (fig. 3), including the regions of putative IgE epitopes. Based on these observations, we hypothesized that Aed a 10.0101 could have a higher IgE reactivity (supported by the ELISA results with the 12 sera showing that the 4 positive IgE sera had higher levels of antibodies to this variant) than Aed a 10.0201 (table 1). These results suggest that Aed a 10.0101 is more represented in the mosquito proteome. Further studies should be performed to evaluate the potential this protein may have for the diagnosis of allergy caused by arthropods, since it seems to have a wide representation of IgE epitopes related to tropomyosin.

In conclusion, we describe that the whole-body extract of *A. aegypti* contains 2 variants of tropomyosin which can mediate IgE cross-reactivity with allergenic tropomyosins from house dust mites, shrimp and the nematode, *A. lumbricoide*s, at different levels. The recombinant molecules of these variants showed different IgE-binding properties. The analysis of the immune response elicited by mosquito tropomyosin and other mosquito antigens and the establishment of whether or not these molecules are present in house dust, or the outdoor environment will help to clarify the allergenic role of *A. aegypti* and to design better schemes for the diagnosis and treatment of mosquito-induced allergies.

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Disclosure Statement

The authors report no conflict of interests in relation to this paper.

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Article 4.

Cantillo JF, Puerta L, Fernández-Caldas E, Subiza JL, Soria I, Lafosse-Marin S, Bohle B.

Immunological characterization of rAed a 8, an important allergen from the mosquito species *Aedes aegypti*.

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Original article

Immunological characterization of rAed a 8, an important allergen from the mosquito species *Aedes aegypti*

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Aedes aegypti, Heat shock cognate-70 protein, mosquito allergy, recombinant allergen.

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ABSTRACT

BACKGROUND: *Aedes aegypti* is the source of saliva as well as somatic allergens. In this study, we performed the immunological characterization of the heat shock cognate-70 allergen Aed a 8, which represents a potential major somatic allergen from *A. aegypti*.

METHODS: Recombinant Aed a 8 (rAed a 8) was expressed in E. coli, purified and its IgE-binding capacity was evaluated by ELISA and immunoblotting using the sera from 14 mosquito skin test positive individuals residing on the tropical island of Martinique. The allergenicity of rAed a 8 was also studied by the Basophil Activation Test and ELISA competition. BALB/c mice were immunized with rAed a 8 and specific IgE, IgG1, IgG2a and IgG3 were measured. Murine IgG1 binding of the whole extract was verified by direct ELISA. T-cell activation was studied in splenocytes stimulated with rAed a 8.

RESULTS: rAed a 8 was expressed as a 74 kDa biologically active protein that reacted with specific IgE in 6 out of 14 (43%) allergic individuals, and induced the activation of basophils. Mice immunized with rAed a 8 produced specific IgE, IgG1, IgG2a and IgG3 antibodies. Murine IgG1 reacted with the natural protein present in the mosquito extract. rAed a 8 induced proliferation of mice splenocytes.

CONCLUSION: rAed a 8 preserved the molecular and immunological capacity of the natural counterpart. The allergen induced a mixed antibody response characterized by the production of Th1 and Th2 related antibodies. Aed a 8 is an important allergen that could be considered in diagnostic tests and in specific-immunotherapy for mosquito allergy.

INTRODUCTION

Sensitization and allergic reactions induced by mosquito allergens have been widely described [1]. Some studies have established that 2.5–47% of individuals with asthma and/or allergic rhinitis are sensitized to mosquito allergens [2-4].

Currently, 4 salivary allergens from *A. aegypti* (Aed a 1, Aed a 2, Aed a 3 and Aed a 4) are deposited in the WHO/IUIS Allergen Nomenclature Sub-Committee. Additionally, we have reported two additional tropomyosins variants [5] and other 5 non-salivary allergens from this mosquito species, including the allergen Heat shock cognate-70, Aed a 8. Aed a 8 reacted with the IgE in 60% of a group of 15 allergic patients from the Caribbean island of Martinique indicating that it may represent a major allergen. This allergen is a member of the Heat shock protein-70 family, chaperones that help in the folding of many proteins maintaining their correct biological function under stress conditions [6]. This protein family is one of the most conserved in evolution [7, 8]. They are allergenic in different species of fungi, cockroach and mite [9-11]. In mosquitoes, this protein is exploited by dengue virus [12] and mosquito-borne flaviviruses (encephalitis virus) [13] to attach and penetrate mosquito cells.

The purification of natural mosquito allergens and their biochemical characterization is a difficult task that may be overcome using biologically active recombinant allergens. The availability of these molecules will help in the understanding of the underlying mechanisms and in the design of new diagnostic and therapeutic tools [14-16].

In this study, we performed the immunological characterization of rAed a 8. The biological active recombinant version of the allergen was obtained by expression in bacteria, purified and its IgE binding capacity evaluated using the sera from a group of allergic patients from the Caribbean, where exposure to mosquitoes is constant. The allergenic activity of this allergen was evaluated by basophil activation test and the immunogenic and antibody response analysed in the splenocytes and sera from immunized mice, respectively.

METHODS

Sera

Serum was obtained from 14 (11 males and 3 females, mean age 11.78 years) allergic patients who attended an outpatient Allergy Clinic in Fort de France, Martinique (Table 1). All individuals were skin tested with a panel of common standardized inhalant allergens and with a commercially available *Aedes communis* extract (Stallergènes, Antony, France). Individuals who developed a wheal > 3 mm to the extracts were considered to have a positive skin prick test (SPT). Serum was obtained after written consent in the context of the approved study entitled: “Etude Epidémiologique des causes Allergiques de l’asthme en Martinique”, funded and approved by “Fonds de’aide a la qualite de soins de ville” from “la Caisse Generale de securite sociale de la Martinique” and the “Comite departemental de la Martinique contre les maladies respiratoires et la tubreculose (CDMMRT)”.

Preparation of extracts and expression and purification of rAed a 8

An *A. aegypti* extract was prepared from lyophilized and defatted mosquitoes (GREER labs, Lenoir, NC, USA), by extraction in PBS, as previously described [5]. For the expression of rAed a 8, the codon optimized nucleotide sequence of the allergen (GenBank: DQ440225.1) was synthesized at GenScript (Township, NJ, USA). The nucleotide sequence was sub-cloned between NdeI and XhoI restriction sites of the multiple cloning site of pET-14b vector (Novagen, Billerica, MA, USA). Plasmid encoding rAed a 8 was used to transform E. coli One Shot BL21 (DE3) (Life Technologies). *E. coli* cells harbouring the expression vector were grown at 37°C in LBA medium. Induction was achieved by addition of 0.5 mM IPTG. After protein expression, the cells were harvested by centrifugation at 10,000 r.p.m. for 10 minutes. The bacterial cell pellet was lysed in lysis buffer (20 mM phosphate buffer pH 7.4, 0.5 M NaCl, 20 mM imidazole) and purified using Ni-NTA resin (Life Technologies) under native conditions, according to the manufacture instructions. Briefly, bacterial lysate was added to the resin for protein binding. After washing the unbounded components, Aed a 8 was eluted with native elution buffer (20 mM phosphate buffer pH 7.4, 0.5 M NaCl, 250 mM imidazole). Protein fractions were analysed by SDS-PAGE and those containing recombinant tropomyosin were pooled, dialysed in 3,500 Da cut-off membranes (Spectra/Por Dialysis membranes) and lyophilized until usage.

Detection of human IgE

Serum specific-IgE to the allergenic extract, or rAed a 8, was measured by ELISA, as described in [5]. Briefly, microplates were coated overnight (ON) at 4ºC with 100 µL of extract (5 µg/mL) or rAed a 8 (0.5 µg/mL), and blocked with PBS with 0.25% tween 20 (PBS-T), containing 1% BSA. Plates were incubated for 2 hours with individual sera, diluted 1:8 in PBS-T containing 1% BSA. IgE binding was detected with horseradish peroxidase-conjugated mouse anti-human IgE Fc (SouthernBiotech) diluted 1:2,000, for 1 hour. The substrate o-Phenylenediamine dihydrochloride (Sigma-Aldrich) was added and reaction was measured at 492 nm after 30 minutes.

Inhibition experiments were performed using a mixture of two sera (Table 1) pre-incubated with the indicated concentrations of inhibitors. Microplates were incubated overnight at 4ºC and the reaction detected as described before. A *P. pratense* extract was used as negative control.

SDS-PAGE and Immunobloting

Two µg of rAed a 8 were separated by SDS-PAGE under denaturing conditions, in 12% continuous polyacrylamide gels at 120 volts using a Mini-PROTEAN II apparatus (Bio-Rad, Hercules, CA, USA). After separation, gels were stained with GelCode Blue stain reagent (Life technologies, Carlsbad, CA, USA). Precision Plus Protein All Blue Standards (Bio-Rad) were used to determine the relative molecular weights of the electrophoresed components. For immunoblotting, proteins were transferred onto 0.45 µm nitrocellulose membrane using a Transblot Semydry Electrophoresis transfer cell (Bio-Rad) at 20 volts for 30 minutes. Non-specific binding was blocked with PBS-T containing 5% BSA, for 45 minutes. Membranes were washed two times with PBS-T, then incubated with serum pool diluted 1:10 in PBS-T containing 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 in a GeneGnome apparatus (Syngene, Cambridge, UK). 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.

Mice immunization

Female BALB/c mice were immunized intraperitoneally (i.p.) with rAed a 8 or PBS adsorbed to Alum hydroxide (Brenntag, Mülheim, Germany). In total, the animals received four injections of 25 µg of antigen adsorbed onto 2 mg Alum, in 250 µL every two weeks. Fifteen days after the last immunization, blood was collected and mice sacrificed and the spleens were removed under aseptic conditions.

Mice antibody measurement

ELISA plates were coated with rAed a 8, or mosquito extract overnight. Afterwards, the sera were added and let to stand overnight. For detection of IgG1, IgG2a and IgG3 sera were diluted 1:500; and for IgE 1:12 and incubated ON. Bound antibodies were detected with rat anti-mouse IgG1, IgG2a, IgG3 and IgE antibodies (BD Pharmingen, San Jose, CA, USA), followed by a HRP-conjugated goat anti-rat IgG (GE Healthcare, Vienna, Austria). ABTS was added and the reaction measured at 405-490 nm.

Lymphoproliferation

Splenocytes (2x105 cells/well) from mice immunized with rAed a 8 or PBS were incubated in round bottom 96 well plates (Nunc) with rAed a 8 (125 ng antigen/well – 7.81 ng antigen/well) or medium alone for 4 days at 37ºC. Concanavalin A (0.5 µg/well; Sigma Aldrich, USA) served as a positive control. During the last 16 hours, 3[H]-labeled thymidine (0.5 mCi/mL) was added. Stimulation indices (SI) were calculated as the ratio between counts per minute (cpm) obtained in cultures with splenocytes plus rAed a 8 and cpm obtained in cultures containing splenocytes and medium alone. A SI of 2 was defined as positive proliferation.

Basophil activation test

PBMCs were purified from blood samples from donors using Ficoll gradient centrifugation. PBMCs were stripped in ice cold lactic acid buffer (0.13 M KCl, 0.05 M NaCl, 0.01 M Lactic acid, pH=3.9). After washing twice with PBS, cells were re-suspended in 80 µL of PBS and 80 µL of serum from an allergic individual and incubated for 1 hour at 37ºC. After washing, re-sensitized cells were incubated with different concentrations of rAed a 8, A. aegypti extract, positive controls (fMLP or anti-IgE) and medium as negative control for 15 minutes. Cells were stained with PE-conjugated anti-human CD63, PerCP conjugated anti-human CD123 and APC-conjugated anti-human CCR3. Basophil activation was expressed as the percentage of CD63+ basophils.

RESULTS

Expression of biologically active rAed a 8

The cDNA coding for Aed a 8 was sub-cloned into an expression vector pET14b and expressed as a soluble His-tag fusion protein in E. coli with a molecular weight of 74 kDa (Figure 1A), which corresponds to the size inferred from its amino acid sequence. The allergen was highly purified by affinity, resulting in a purity higher than 90%. Two low intensity bands were observed at around 72 and 50 kDa. The immunoblotting showed that the band of 74 kDa reacted with the IgE from an allergic individual and weak reactivity was detected for the 72 and 50 kDa bands, suggesting that they may correspond to degradation products (Figure 1B).

Table 1. Clinical details and sensitization profiles of 14 allergic patients sensitized to *A. aegypti,* used in the study.

Serum	Age (years)	Gender	Diagnosis	Total IgE IU/mL	Specific IgE against *A. aegypti* extract (O.D.)	Specific IgE against rAed a 8 (O.D.)
1*	5	M	Rhinitis	324	0.319	0.593
2	13	M	Rhinitis, Conjunctivitis	2832	0.186	0.083
3	6	M	Rhinitis, Conjunctivitis	552	0.530	0.074
4	4	F	Rhinitis, Conjunctivitis	2604	1.221	0.067
5*	3	M	Rhinitis	8.7	1.982	0.151
6*	11	M	Asthma, Rhinitis, Conjunctivitis	2095	1.679	0.120
7*	11	M	Rhinitis, Conjunctivitis	611	0.111	0.093
8	13	M	Rhinitis, Conjunctivitis	1199	0.795	0.070
9*	8	M	Asthma, Rhinitis, Conjunctivitis	1659	0.511	0.129
10	8	M	Rhinitis	626	0.165	0.082
11	37	F	Rhinitis, Conjunctivitis	58	0.332	0.063
12	15	M	Asthma, Rhinitis, Conjunctivitis	1246	1.240	0.068
13*	27	F	Rhinitis	545.5	0.355	3.990
14	4	M	Asthma, Conjunctivitis	2187	0.458	0.066
Mean	11.78	78.6% M		1182	0.706	0.403

*Sera from these patients were considered as rAed a 8-specific IgE positive

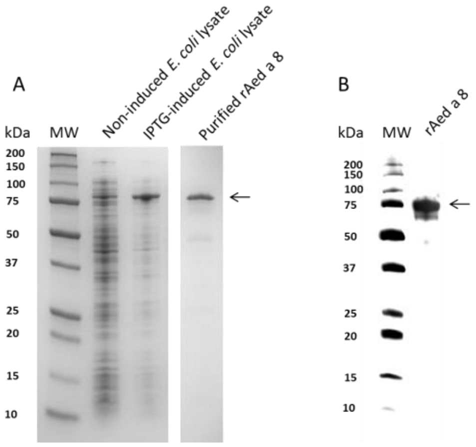

Figure 1. Expression, purification and IgE-binding of rAed a 8. (A) SDS-PAGE of supernatants from non-induced and induced cultures showing a 74 kDa band of interest. More than 90% of purity was achieved after affinity purification. (B) Immunoblotting of rAed a 8 with an individual serum from an allergic patient. MW: Molecular weight marker

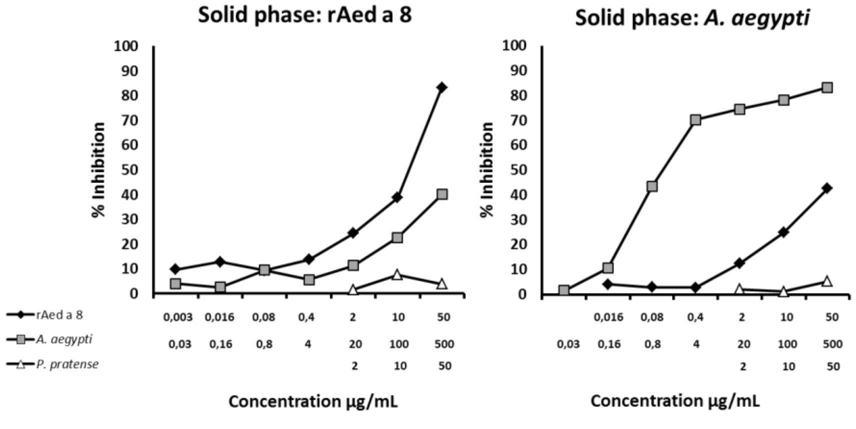

Figure 2. ELISA inhibition assays between A. aegypti extract and rAed a 8. Microplates coated with rAed a 8 or whole extract were incubated with a mixture of 2 sera previously adsorbed with different concentrations rAed a 8 or whole mosquito extract. P. pretense extract was used as negative control.

rAed a 8 binds IgE from 43% of HDM-allergic individuals and has similar antibody reactivity as natural Aed a 8

We further evaluated the IgE frequency of reactivity of rAed a 8 in a group of 14 allergic patients suffering asthma, rhinitis, and/or conjunctivitis by ELISA. rAed a 8 was recognized by 6 of the 14 patients (42.8%) (Table 1). By ELISA inhibition we compared the inhibition capacity of rAed a 8 with the whole extract (Figure 2). When the whole extract was on the solid phase, rAed a 8 inhibited in 42% the IgE reactivity of the sera against the extract. When rAed a 8 was on the solid phase, the whole extract produced a similar degree of inhibition (Figure 2).

rAed a 8 induces dose-dependent basophil activation

The allergenic activity of rAed a 8 was analysed using stripped basophils sensitized with serum from one allergic patient with Aed a 8-specific IgE. The upregulation of CD63 was measured after incubating the cells with increasing concentrations of rAed a 8 or *A. aegypti* extract (Figure 3). rAed a 8 induced a dose-dependent up-regulation of the marker, while the extract produced higher activation at 1 ng/mL and 0.1 ng/mL. The optimum concentration for rAed a 8 was 1000 ng/mL and for the extract 0.1 ng/mL.

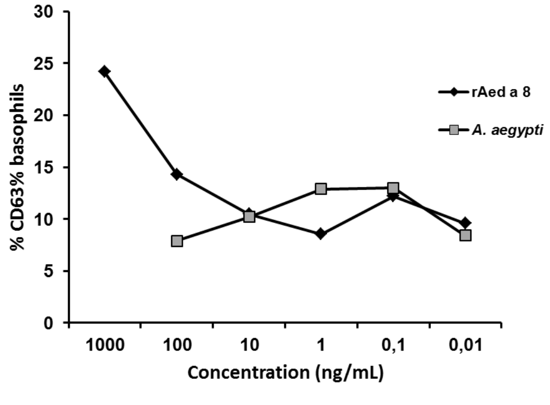

Figure 3. Basophil activation test with rAed a 8 and *A. aegypti* extract. Stripped basophils sensitized with the IgE from the sera of an *A. aegypti* sensitized patient were stimulated with different concentrations of rAed a 8 or whole mosquito extract. Activation of basophils was expressed as the percentage of $CD63^+$ basophils.

Murine immune response of rAed a 8 showed strong immunogenic capacity

Splenocytes were stimulated with different concentrations of rAed a 8 to assess the T-cell reactivity. Splenocytes isolated from rAed a 8-immunized mice proliferated upon stimulation to different concentrations of the recombinant allergen. No proliferation was observed in the splenocytes from the mice immunized with PBS (Figure 4).

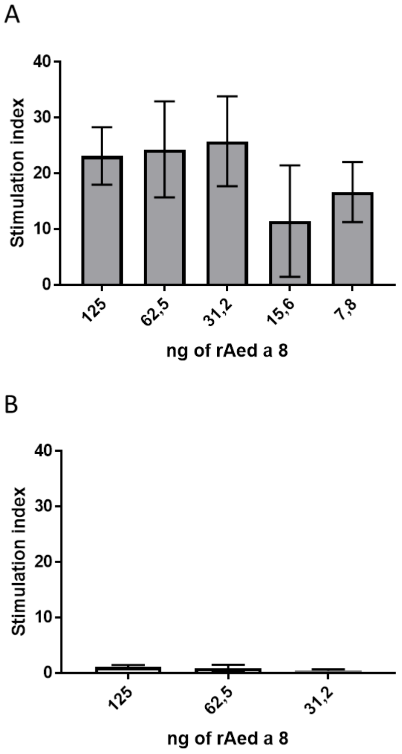

Figure 4. T-cell proliferation to rAed a 8 in mice splenocytes. Splenocytes from mice immunized with rAed a 8 or PBS were stimulated with different concentrations of rAed a 8. Proliferation was measured by means of the incorporation of 3[H]-labeled thymidine.

BALB/c mice immunized with rAed a 8 produced specific IgE, IgG1, IgG2a and IgG3. In the serum of the mice immunized with PBS the levels of these antibodies were significantly lower (Figure 5A). The antibody response was characterized by a high production of IgG2a. Furthermore, total IgG produced by the mice immunized with rAed a 8 bound the natural allergen contained in the mosquito extract (Figure 5B), indicating that they recognized its natural counterpart.

DISCUSSION

Several studies have suggested that the mosquito species *A. aegypti* contains somatic allergens [1, 5, 17]. The WHO/IUIS Allergen Nomenclature Sub-Committee has accepted the inclusion of 6 novel allergens from *A. aegypti* which react with specific IgE from patients with asthma, rhinitis and/or conjunctivitis, including the Heat shock cognate-70 allergen, Aed a 8. However, the role of this, and other allergens, in the onset and natural course of allergic responses is still under study. In this work, we obtained and purified a biologically active recombinant allergen, rAed a 8, by expression in *E. coli*.

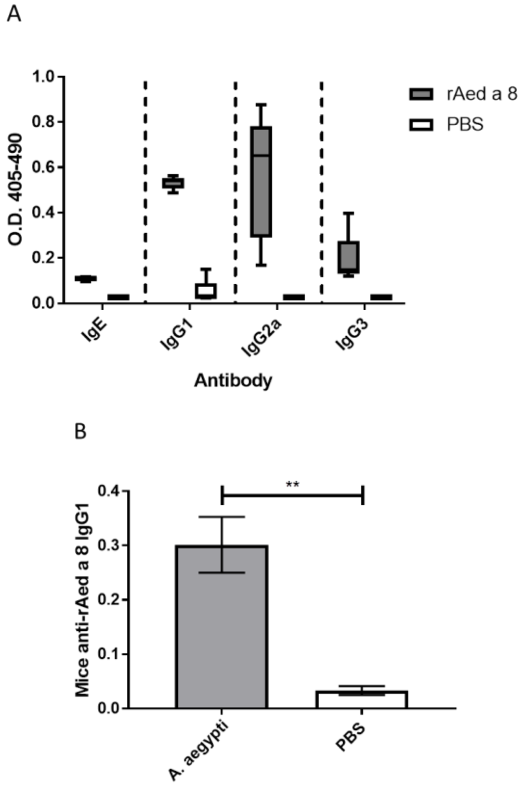

Figure 5. Antibody response to rAed a 8 in BALB/c mice. (A) Specific-IgE and IgG subclasses of the mice. (B) Specific-IgG1 from mice immunized with rAed a 8 reactivity to the whole body mosquito extract.

This molecule showed specific IgE binding capacity and was able to inhibit IgE binding to the natural allergen extract. Furthermore, rAed a 8 induced the activation of basophils and showed strong immunogenicity in mice. The immune response induced by this allergen was characterized by the production of high levels of IgG subclasses, suggesting a deviation of the response to a Th1 phenotype.

rAed a 8 was expressed in *E. coli* and purified as a single band of approximately 74 kDa, which corresponded to the theoretical molecular weight inferred by its aminoacid sequence. Using the sera from allergic patients (Table 1) and the sera from the immunized mice (Figure 5B) we demonstrated that rAed a 8 has similar antibody binding capacity compared to the natural allergen contained in the mosquito extract. In ELISA inhibition experiments we demonstrated that Aed a 8 accounted for 40% of the total IgE-binding of the extract.

Allergens from the Heat shock protein-70 family have been reported in several fungi species, such as *Alternaria alternata* (Alt a 3) [10], *Malassezia sympodialis* (Mala s 10) [18] and *Penicillium citrinum* (Pen c 19) [19], with an IgE-binding frequency of 5% for Alt a 3 and 41% to 69% in the other mold species. In mites, heat shock proteins have been identified in *D. farinae* (Der f 28) [9]; 68.3% of a group of 41 house dust mite allergic patients presented serum specific-IgE to Der f 28. However, when used as skin prick test reagent, only 11.5% of the patients were positive [20]. Tyr p 28, from *Tyrophagus putrescentia*e, reacted with specific IgE in 47% of the allergic patients. In our study, we observed an IgE-binding frequency of 43%; however, the number of patients was low, indicating that further studies in representative allergic populations should be performed to define whether this is a major, or a minor allergen.

One important requirement of the recombinant allergens is their capacity to mimic their natural counterparts. In our study, we demonstrated that rAed a 8 preserved IgE-binding. Furthermore, the IgG1 produced by mice immunized with rAed a 8, recognized the natural counterpart contained in the mosquito extract (Figure 5B). rAed a 8 induced the activation of basophils (Figure 3) and lymphoproliferation of mice cells (Figure 4). These results suggest that the natural and recombinant Aed a 8 share B-cell epitopes and that rAed a 8 conserves its allergenic and immunogenic capacity.

Interestingly, the BALB/c mice immunized with rAed a 8 produced low levels of IgE and high levels of IgG subclasses, specially IgG2a. This result was unexpected because the presence of IgG2a is an indicator of a Th1 responses. However, there are several studies which indicate that mosquito allergens, including Aed a 8, may play a protective role in the allergic response. The natural course of mosquito bite allergy indicates that the patients experience allergen desensitization, accompanied with a high production of total IgG and IgG4 [21]. It has also been suggested that *A. aegypti* bites trigger an atypical allergic reaction in a mouse model of mosquito bite allergy, consisting in a mixed Th1/Th2 phenotype characterized by the production of high levels of IgG2a [22]. Furthermore, in a mouse model of allergy, oropharyngeal injection of exogenous HSP70 resulted in significant suppression of the inflammatory process during the acute phase of allergic inflammation of the airways [23]. These data suggest that the HSP70 family may have an immunosuppressive activity. It seems that rAed a 8 also has this capacity and it represents a potential candidate to further evaluate its potential use in specific immunotherapy.

In conclusion, we have cloned and expressed a 74 kDa rAed a 8, which conserves the antibody-binding, allergenic and immunogenic capacity. The antibody response induced in immunized mice indicates that this allergen may have a potential immune regulatory role. rAed a 8 represents an important allergen recognized by more than 40% of the mosquito allergic population. It should be further investigated and considered for diagnosis and immunotherapy of mosquito induced allergies.

Conflict of Interest: none.

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C. Cross-reactivity between *A. aegypti* and other arthropods

In this part of the thesis, we explored cross-reactivity between *A. aegypti* and other arthropod allergens at different levels. The classical concept of cross-reactivity refers to a phenomenon in which the antibodies raised against a molecule bind to a different but structurally similar molecule. Under this concept, cross-reactivity may have clinical consequences. In allergy diagnosis, cross-reactivity may lead to false positives; however, for the allergen specific immunotherapy the application of a single molecule may desensitize the patient to a broad spectrum of similar molecules. In addition, the capacity of a molecule to cross-react with specific IgE, previously produced against a primary sensitizer, may induce allergic symptoms. Studies have shown that cross-reactivity may also involve T-cell epitopes, which, together with the T-cell cross-reactive B-cell epitopes, are necessary in the exacerbation of the allergic response.

Initially, cross-reactivity between mosquito and other arthropod extracts was evaluated in ELISA experiments and the implicated components detected in immunoblot inhibition experiments. More than 10 proteins were identified, and four of them corresponded to Odorant Binding Protein, Mitochondrial cytochrome C, Cyclophilin (PPIase), an unknown protein “AAEL001668-PA” and tropomyosin.

Furthermore, the humoral and cellular cross-reactivity between *A. aegypti* tropomyosins (rAed a 10.0101 and rAed a 10.0201) and rDer p 10 was analysed by ELISA, Basophil activation test, ELISA and Basophil activation inhibition assays, and lymphoproliferation using splenocytes from immunized BALB/c mice. The results showed that the three tropomyosins cross-react at the different levels and that the degree of cross-reactivity depended on the degree of sequence homology.

For further information see the following papers:

Article 5.

Cantillo JF, Puerta L, Lafosse-Marin S, Subiza JL, Caraballo L, Fernández-Caldas E. Allergens Involved in The Cross-reactivity of *A. aegypti* and Other Arthropods.

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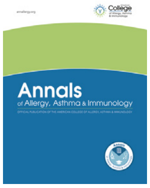

Allergens involved in the cross-reactivity of *Aedes aegypti* with other arthropods

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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.

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 Inmunotek 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	*Aedes aegypti*	*Dermatophagoides pteronyssinus*	*Dermatophagoides farinae*	*Blomia tropicalis*	*Periplaneta americana*	*Litopenaeus vannamei*	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)

[a] Specific 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.

[b] Monosensitized to *Aedes aegypti*.

[c] Sensitized to *A aegypti* and *Periplaneta americana*.

[d] Serum samples from these patients were used for preparing the pool of 7 serum samples.

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

Patient no.	Diagnosis	Mosquito SPT	*Dermatophagoides pteronyssinus* SPT	*Blomia tropicalis* 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.
[a] Plus sign indicates positive test result; minus sign, negative test result.

Determination of Specific IgE

Plastic Microlon, 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 washing, 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 Proteineer 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 (*Blattela 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 μg/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).

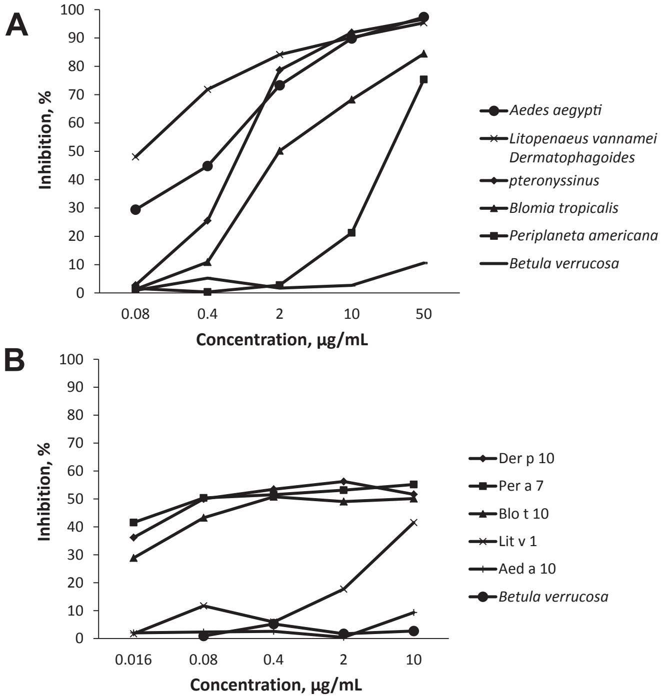

Figure 1. Enzyme-linked immunosorbent assay inhibition. Microplate wells coated with *Aedes aegypti* extract were incubated with pooled sera previously adsorbed with different concentrations of arthropod extracts (A) or recombinant tropomyosins (B) as inhibitors.

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 μg/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 tropomyosin[32] 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 reveasled 81.1% identity in the amino acid sequences (Fig 3). We further conducted a comparative structural analysis of PPIase with other related allergens.

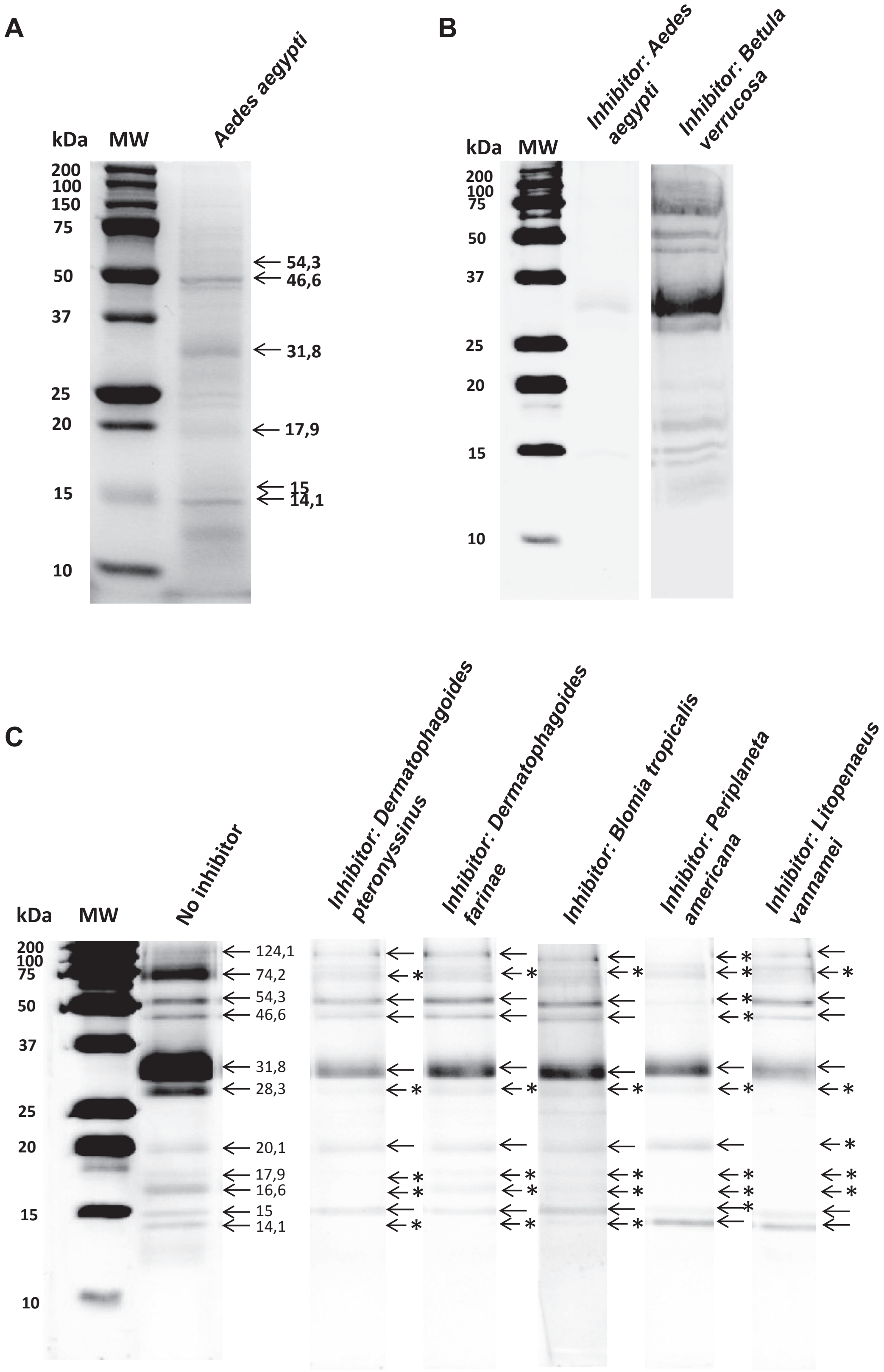

Figure 2. Coomassie blue–stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (A) and immunoblot inhibition of *Aedes aegypti* extract (B and C). Noninhibited bands are indicated with arrow and inhibited bands with arrow plus asterisk. MW, molecular weight.

Structural Analysis of PPIase From A aegypti

The modeled PPIase 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 cycliphilins, which contain 7. Multiple sequence alignment of PPIase from *A aegypti* with cyclophilins reported as allergens (Fig 5A) reveals a high

Table 3
Proteomic and Homology Search Results of New IgE Reactive Bands Isolated From *Aedes aegypti*

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

Abbreviations: MS, mass spectroscopy; MW, molecular weight; NF, not found.

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 11 from *Aspergillus fumigatus*, Mala d 6 from *Malassezia dermatis*, and Bet v 7 from *Betula verrucosa*. Two highly conserved residues (^{99}SMANAGPNTNGSQFFI^{113} and ^{121}WLDDRHVVFG^{130} in the mosquito PPIase 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 PPIase, 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 PPIase 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* PPIase.

Discussion

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 allergenic symptoms has not yet been defined, and the results of this study suggest that further research is clearly needed to clarify this issue.

Several studies have revealed a prevalence of sensitization to mosquito from 4% to 47.8%.[42–45] The frequency of IgE reactivity to *A aegypti* found in this study (64.7%) is one of the highest reported and is similar to the prevalence of sensitization to mites in allergic individuals. Although a small number of serum samples were 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

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PPIase      MGLPRCFFDLTADNAPLGRVVIELRTDVTPKTCENFRALCTGEKGFGYKGSTFHRVIPNF
Der f 29    MALPRVFFDIAADNQPLGRIVIELRSDVVPKTAENFRALCTGEKGFGFKSSSFHRIIPNF
            *.*** ***::*** ****:*****:**.***.**************:*.*:***:****

PPIase      MCQGGDFQNHNGTGGKSIYGNKFEDENFILRHTGPGIMSMANAGPNTNGSQFFITTVKTS
Der f 29    MIQGGDFTNHNGTGGKSIYGNKFADENFTLQHTGPGIMSMANAGPNTNGSQFFITTVKTT
            * ***** *************** **** *:****************************:

PPIase      WLDDRHVVFGSVVEGMDVIRKVESYGNQSGKTSKQIVVSNCGQL
Der f 29    WLDGKHVVFGSVVEGMDIVKKVESYGSQSGKPSKKVTIANCGQL
            ***.:************:::******.****.**::.::*****
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Figure 3. Amino acid sequence alignment of peptidyl-prolyl cis-trans isomerase (PPIase) from *Aedes aegypti* and its homologue allergen, Der f 29, from the mite *Dermatophagoides farinae*.

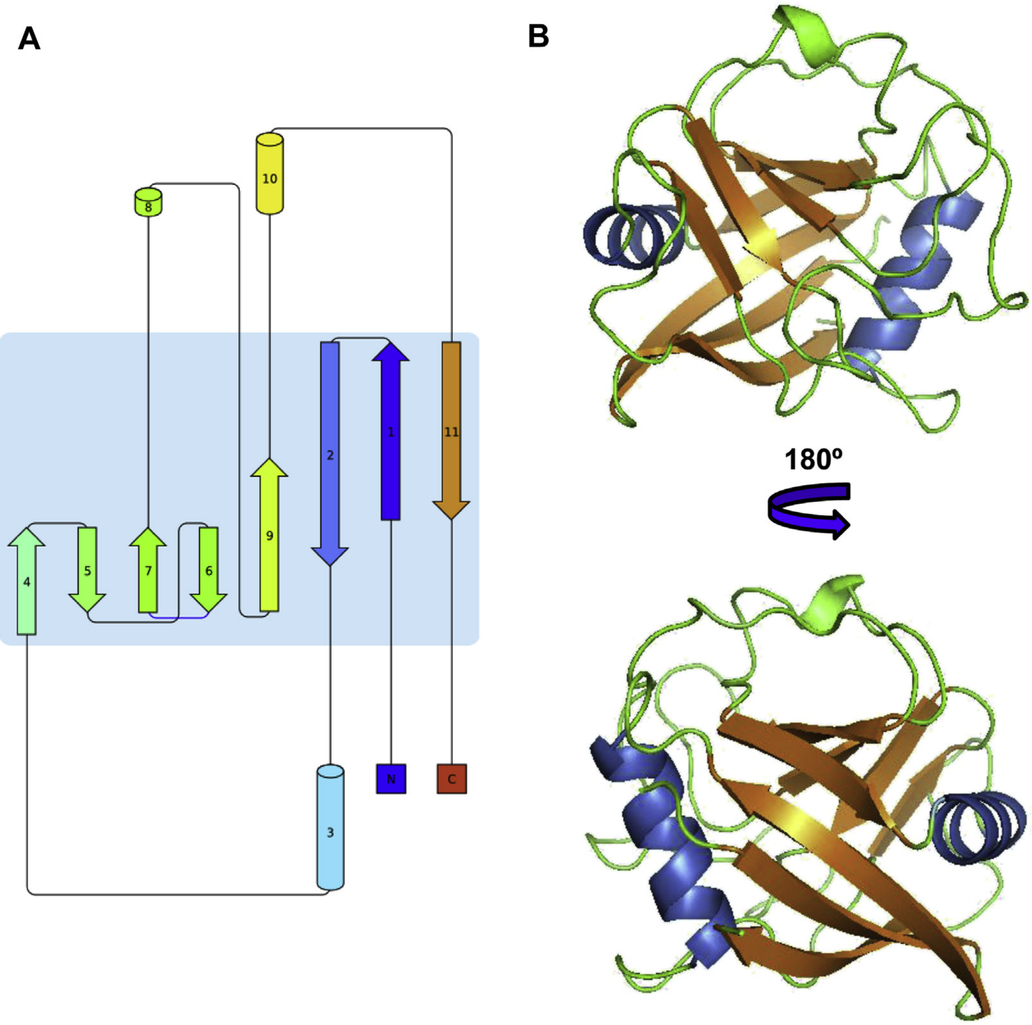


Figure 4. Structural characteristics of peptidyl-prolyl cis-trans isomerase from *Aedes aegypti*. (A) Topologic diagram showing the secondary structure elements. (B) Modeled 3-dimensional structure showing a β-barrel of 8 antiparallel β-strands and 2 surrounding α-helices.

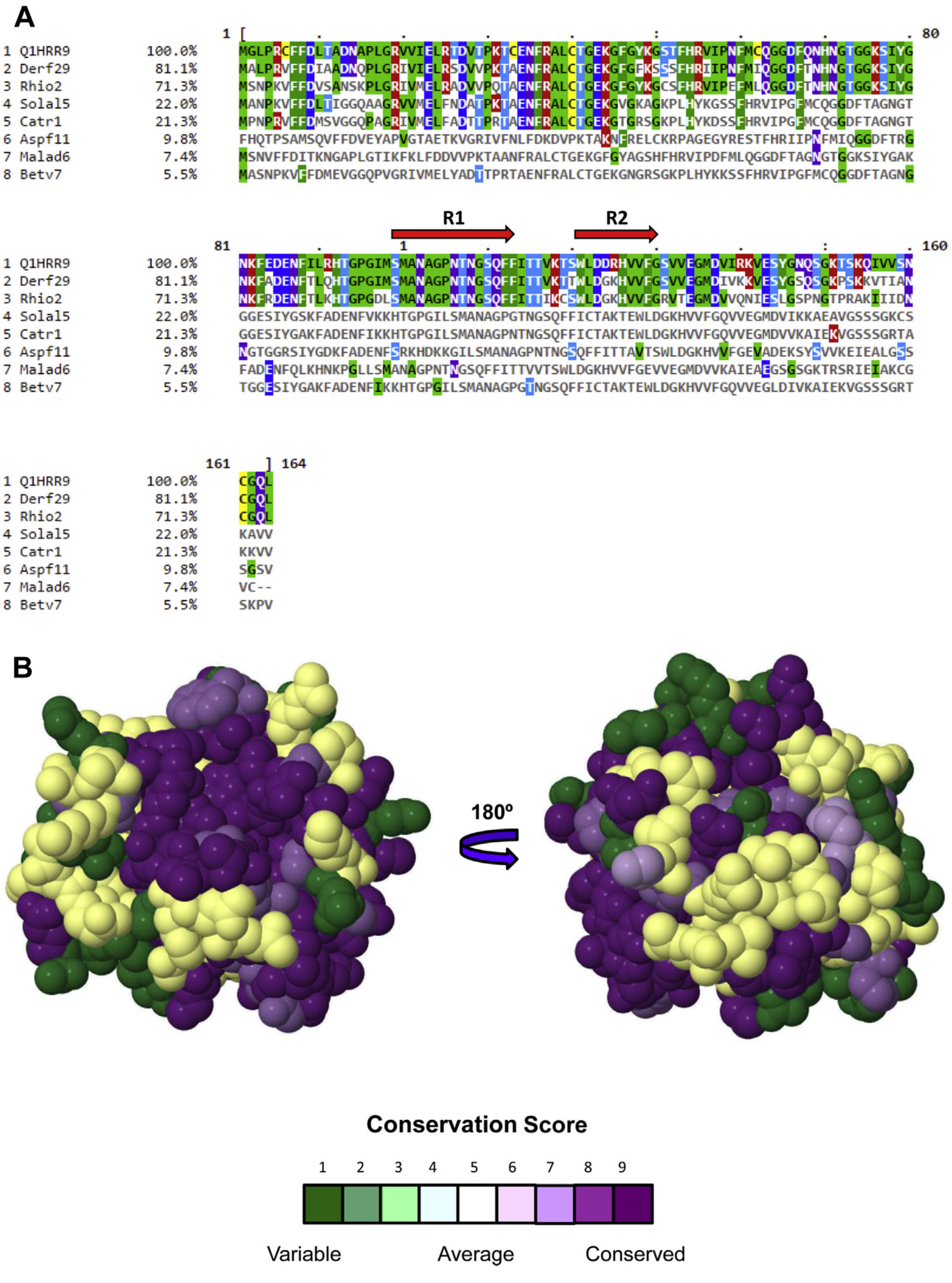
glands contain the allergens involved in allergic skin reactions caused by mosquito bites.[2,4,7]

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 immunoblotting[51] 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



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                            1 [         .         .         .         .         :         .         .         . 80
1 Q1HRR9        100.0%        MGLPRCFFDLTADNAPLGRVVIELRTDVTPKTCENFRALCTGEKGFGYKGSTFHRVIPNFMCQGGDFQNHNGTGGKSIYG
2 Derf29         81.1%        MALPRVFFDIAADNQPLGRIVIELRSDVVPKTAENFRALCTGEKGFGFKSSSFHRIIPNFMIQGGDFTNHNGTGGKSIYG
3 Rhio2          71.3%        MSNPKVFFDVSANSKPLGRIVMELRADVVPQTAENFRALCTGEKGFGYKGCSFHRVIPEFMLQGGDFTNHNGTGGKSIYG
4 Solal5         22.0%        MANPKVFFDLTIGGQAAGRVVMELFNDATPKTAENFRALCTGEKGVGKAGKPLHYKGSSFHRVIPGFMCQGGDFTAGNGT
5 Catr1          21.3%        MPNPRVFFDMSVGGQPAGRIVMELFADTTPRTAENFRALCTGEKGTGRSGKPLHYKDSSFHRVIPGFMCQGGDFTAGNGT
6 Aspf11          9.8%        FHQTPSAMSQVFFDVEYAPVGTAETKVGRIVFNLFDKDVPKTAKNFRELCKRPAGEGYRESTFHRIIPNFMIQGGDFTRG
7 Malad6          7.4%        MSNVFFDITKNGAPLGTIKFKLFDDVVPKTAANFRALCTGEKGFGYAGSHFHRVIPDFMLQGGDFTAGNGTGGKSIYGAK
8 Betv7           5.5%        MASNPKVFFDMEVGGQPVGRIVMELYADTTPRTAENFRALCTGEKGNGRSGKPLHYKKSSFHRVIPGFMCQGGDFTAGNG

                                                  R1                     R2
                           81         .         1         .         .         .         .         :         . 160
1 Q1HRR9        100.0%        NKFEDENFILRHTGPGIMSMANAGPNTNGSQFFITTVKTSWLDDRHVVFGSVVEGMDVIRKVESYGNQSGKTSKQIVVSN
2 Derf29         81.1%        NKFADENFTLQHTGPGIMSMANAGPNTNGSQFFITTVKTTWLDGKHVVFGSVVEGMDIVKKVESYGSQSGKPSKKVTIAN
3 Rhio2          71.3%        NKFRDENFTLKHTGPGDLSMANAGPNTNGSQFFITTIKCSWLDGKHVVFGRVTEGMDVVQNIESLGSPNGTPRAKIIIDN
4 Solal5         22.0%        GGESIYGSKFADENFVKKHTGPGILSMANAGPGTNGSQFFICTAKTEWLDGKHVVFGQVVEGMDVIKKAEAVGSSSGKCS
5 Catr1          21.3%        GGESIYGAKFADENFIKKHTGPGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGQVVEGMDVVKAIEKVGSSSGRTA
6 Aspf11          9.8%        NGTGGRSIYGDKFADENFSRKHDKKGILSMANAGPNTNGSQFFITTAVTSWLDGKHVVFGEVADEKSYSVVKEIEALGSS
7 Malad6          7.4%        FADENFQLKHNKPGLLSMANAGPNTNGSQFFITTVVTSWLDGKHVVFGEVVEGMDVVKAIEAEGSGSGKTRSRIEIAKCG
8 Betv7           5.5%        TGGESIYGAKFADENFIKKHTGPGILSMANAGPGTNGSQFFICTAKTEWLDGKHVVFGQVVEGLDIVKAIEKVGSSSGRT

                          161    ] 164
1 Q1HRR9        100.0%        CGQL
2 Derf29         81.1%        CGQL
3 Rhio2          71.3%        CGQL
4 Solal5         22.0%        KAVV
5 Catr1          21.3%        KKVV
6 Aspf11          9.8%        SGSV
7 Malad6          7.4%        VC--
8 Betv7           5.5%        SKPV
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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.

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Article 6.

Cantillo JF, Puerta L, Fernández-Caldas E, Subiza JL, Soria I, Woehrl S, Ebner C, Keller W, Resch Y, Vrtala S, Bohle B. Molecular and immunological cross-reactivity between mite and mosquito tropomyosins.

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Original article

Molecular and immunological cross-reactivity between mite and mosquito tropomyosins

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ABSTRACT

Background: *Dermatophagoides pteronyssinus* and *Aedes aegypti* are important sources of allergens, including tropomyosin. The functional and clinical relevance of this cross-reactivity is still a matter of debate. In this study we analysed the molecular and cellular cross-reactivity among tropomyosins from *A. aegypti* (Aed a 10.0101 and Aed a 10.0201) and *D. pteronyssinus* (Der p 10).

Methods: Sera from 15 Austrian HDM-allergic patients sensitized to Der p 10 were tested for cross-reactivity with rAed a 10.0101 and rAed 10.0201, by ELISA, basophil activation test and inhibition assays. BALB/c mice were immunized with Aed a 10.0101 or Aed a 10.0201 and IgE and IgG antibodies measured. The cellular cross-reactivity and T-cell epitope mapping were analysed in splenocytes stimulated with the tropomyosins.

Results: Der p 10-sensitized patients displayed IgE-reactivity to tropomyosins from *A. aegypti*. Aed a 10.0101 produced a more pronounced inhibition of the IgE-binding to Der p 10 and was also more potent in activating basophils than Aed a 10.0201. Mice immunized with Aed a 10.0101 and Aed a 10.0201 produced specific IgE, IgG1, IgG2a and IgG3 antibodies which cross-reacted with Der p 10. All tropomyosins induced proliferation of mice splenocytes. Five regions in the amino acid sequence were identified to contain cross-reactive T-cell epitopes.

Conclusion: Tropomyosins from *D. pteronyssinus* and *A. aegypti* showed humoral and cellular cross-reactivity, involving 5 potential T cell-activating regions. The cross-reactive potential increases with their amino acid sequence homology. A more detailed characterization of tropomyosins will contribute to elucidate the clinical impact of tropomyosin cross-reactivity in mites, mosquitoes and other arthropods.

INTRODUCTION

The inhalation of *Dermatophagoides pteronyssinus* allergens is one of the main causes of IgE-mediated allergies worldwide (1). The mosquito species *Aedes aegypti* is also a source of several allergens (2, 3). However, its role in the onset or exacerbation of allergic diseases is still under evaluation. HDM and mosquitoes are evolutionarily related as part of the Phylum Arthropoda and IgE cross-reactivity between them has been demonstrated (4). The presence of pan-allergens, such as tropomyosin, may partially explain this phenomenon. A high degree of cross-reactivity has been demonstrated between HDM and crustaceans (5), mollusks (6) and insects (7), with tropomyosin being one of the potential allergens involved.

Tropomyosin is a pan-allergen (8) that belongs to a two-stranded alpha-helical coiled coil proteins family, which can induce allergic sensitization by ingestion (seafood), inhalation (mites, cockroaches), or parasite infection (9, 10). In developed countries, the IgE frequency of sensitization to tropomyosin is low (11, 12). However, in Africa and South America, the prevalence is approximately 50% (13, 14). Several tropomyosin allergens have been described in mites (15), helminths (16) and insects (17), including mosquitoes (3).

The molecular aspects of allergenic cross-reactivity among the different tropomyosins have been studied. Ayuso, R. et al. (18, 19) described eight peptides which correspond to five putative cross-reactive IgE-binding epitopes sharing from 56% to 98% of homology with tropomyosins from shrimp, lobster, house dust mite, and cockroach. These regions coincided with five IgE binding mimotopes described by Leung N. et al (20). T-cell epitopes from shrimp and cockroach tropomyosins restricted to multiple MHC class II alleles have also been described (21, 22). However, to the best of our knowledge cross-reactivity at the T-cell epitope level has not been described.

It has been demonstrated that natural A*. aegypti* tropomyosin highly cross-react with HDM tropomyosin. *A. aegypti* produces two tropomyosin variants; Aed a 10.0101 and Aed a 10.0201 (3), which slightly differ in their primary structure. Aed a 10.0101 has higher homology to other allergenic tropomyosins and higher conservation of the putative IgE-binding epitopes described by Ayuso, R. et al. However, a greater number of substitutions were observed in Aed a 10.0201. Based on ELISA competition experiments and sequence analyses, we suggested that Aed a 10.0101 contains a higher number of IgE binding epitopes, is more allergenic and may be more cross-reactive than Aed a 10.0201 (3).

In this study, we analysed the humoral and cellular cross-reactivity between recombinant HDM tropomyosin (rDer p 10) and *A. aegypti* tropomyosins (rAed a 10.0101 and rAed a 10.0201). The humoral cross-reactivity was tested by immunoassays using human IgE and murine antibodies, and the basophil activation test. The cellular cross-reactivity and T-cell epitope mapping were analysed in splenocytes from mice immunized with mosquito-derived tropomyosins.

MATERIALS AND METHODS

Sera

In total, 15 patients (9 female, 6 male, median age of 25 years, Table 1) allergic to HDM were included. All individuals suffered from perennial rhinoconjunctivitis and showed positive skin prick tests to HDM extract (ALK Abello, Hørsholm, Denmark), as well as IgE specific for HDM and Der p 10 as determined by ImmunoCAP (Table 1).

Allergens

Recombinant (r) Der p 10 was expressed as described. Whole body extract of *A. aegypti*, and recombinant tropomyosins of *A. aegypti*, Aed a 10.0101 and Aed a 10.0201, termed rAed a 10.01 and rAed a 10.02 in the following, were produced as described (3).

Circular dichroism

Far UV CD spectra of rDer p 10 (280 µg/ml 0.1M PBS), rAed a 10.01 (250 µg/ml 0.1M Tris-HCl,) and Aed a 10.02 (500 µg/ml 0.1M PBS) were collected on a Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) using a 1mm path length quartz cuvette. Measurements were done between 250 to 190 nm, with 0.5 nm resolution at a scanning speed of 50 nm/min. Three independent measurements were recorded and averaged for each spectral point. The final spectra were baseline corrected by subtracting the corresponding buffer spectrum. Results were expressed as the mean residue ellipticity [θ] at a given wavelength

Detection of human IgE

ELISA plates (Nunc Maxisorp, Thermo Fisher Scientific, Waltham, MA, USA) were coated overnight (ON) at 4ºC with extract (250 µg/ml) or recombinant allergens (0.5 µg/ml), in carbonate buffer (pH=9.6). After washing twice with PBS containing 0.05% Tween 20 (PBS-T), non-specific binding was blocked by incubation for 6 hours at RT with PBS-T supplemented with 1% HSA. Plates were incubated ON at 4ºC, with the sera diluted 1:4 in PBS-T containing 1% HSA. After washing, bound IgE was detected with alkaline-phosphatase conjugated anti-human IgE Ab (BD Pharmingen, San Jose, CA, USA) diluted 1:2,000 in PBS-T containing 1% HSA, for 60 minutes at 37ºC and 60 minutes at 4ºC. After washing, the substrate PNPP was added and the reaction was measured at 405-550 nm. All experiments were performed in duplicate. Sera from three non-allergic individuals were used as negative controls. The mean values from these samples plus five standard deviations were used as the cut off for positive IgE levels. For inhibition experiments sera were pre-incubated with the indicated concentrations of the recombinant tropomyosins.

Mice immunization

Female BALB/c mice were immunized intraperitoneally (i.p.) with either *A. aegypti* extract, rAed a 10.01 or rAed a 10.02, or PBS adsorbed to Alum hydroxide (Brenntag, Mülheim, Germany). In total, the animals received four i.p. injections of 25 µg of antigen adsorbed to 2 mg Alum, in 250 µL every two weeks. Fifteen days after the last immunization, blood was collected and mice were sacrificed and the spleens were removed under aseptic conditions.

Detection of murine antibody responses

ELISA plates were coated and saturated as described above. For detection of IgG1, IgG2a and IgG3 sera were diluted 1:500; and for IgE 1:12 and incubated ON. Bound antibodies were detected with rat anti-mouse IgG1, IgG2a, IgG3 and IgE antibodies (BD Pharmingen), followed by a HRP-conjugated goat anti-rat IgG (GE Healthcare, Vienna, Austria). ABTS was added and the reaction was measured at 405-490 nm.

Lymphoproliferation

Splenocytes (2x105 cells/well) from mice immunized with rAed a 10.01 and Aed a 10.02 were incubated in round bottom 96 well plates (Nunc) with rDer p 10, Aed a 10.01, Aed a 10.02 (100 ng antigen/well – 12.5 ng antigen/well) or medium alone for 4 days at 37ºC. Concanavalin A (0.5 µg/well; Sigma Aldrich, USA) served as positive control. In addition, splenocytes were incubated with 28 overlapping synthetic 20-mer peptides (1 µg/well) representing the complete amino acid sequence of Aed a 10.01 (Thermo Fisher Scientific, Waltham, MA, USA). During the last 16 hours 3[H]-labeled thymidine (0.5 mCi/mL) was added. Stimulation indices (SI) were calculated as the ratio between counts per minute (cpm) obtained in cultures with splenocytes plus tropomyosins and cpm obtained in cultures containing splenocytes and medium alone. A SI of 2 was defined as positive proliferation.

Basophil activation test

PBMCs were purified from blood samples from donors using Ficoll gradient centrifugation. PBMCs were stripped in ice cold lactic acid buffer (0.13 M KCl, 0.05 M NaCl, 0.01 M Lactic acid, pH=3.9). After washing twice with PBS, cells were re-suspended in 80 µL of PBS and 80 µL of sera from HDM-allergic individuals and incubated for 1 hour at 37ºC. After washing, re-sensitized cells were incubated with different concentrations of each tropomyosin, positive controls (fMLP or anti-IgE) and medium as negative control for 15 min. Cells were stained with PE-conjugated anti-human CD63, PerCP-conjugated anti-human CD123 and APC-conjugated anti-human CCR3. Basophil activation was expressed as the percentage of CD63+ basophils.

For inhibition experiments, allergens were pre-incubated with sera from mice immunized with *A. aegypti* antigens (rAed a 10.01, rAed a 10.02 and extract) and used for stimulation of re-sensitized basophils, as described.

Table 1. Clinical details and sensitization profiles of 15 HDM with specific IgE against Der p 10, used in the study.

Patient No.	**Age [y]**	**Sex**	**Specific IgE [IU/mL]**		**Specific IgE (O.D.)**		
			HDM	**rDer p 10**	**rDer p 10**	**rAed a 10.0101**	**rAed a 10.0201**
1	51	F	41	1.07	0.263	0.130	0.236
2	9	M	385	6.30	0.857	0.778	0.368
3	8	M	1156	16.8	1.961	1.924	1.215
4	27	F	443	n.d.	1.141	0.919	1.015
5	58	M	763	1.98	0.322	0.279	0.219
6	30	F	694	6.96	0.871	0.860	0.314
7	30	F	33	11.2	1.279	1.282	1.073
8	10	M	303	n.d.	>2.500	>2.500	>2.500
9	25	F	242	2.11	0.385	0.306	0.247
10	64	F	54	55.1	>2.500	>2.500	0.851
11	12	M	14.9	27.5	0.755	0.536	0.331
12	7	F	4.07	10.6	0.444	0.483	0.161
13	15	F	46.9	57.0	1.894	2.081	0.499
14	8	F	0.37	1.54	0.114	0.113	0.092
15	40	M	1.47	7.71	0.353	0.460	0.310

M: Male; F: Female; IU: International units; n.d.: not determined

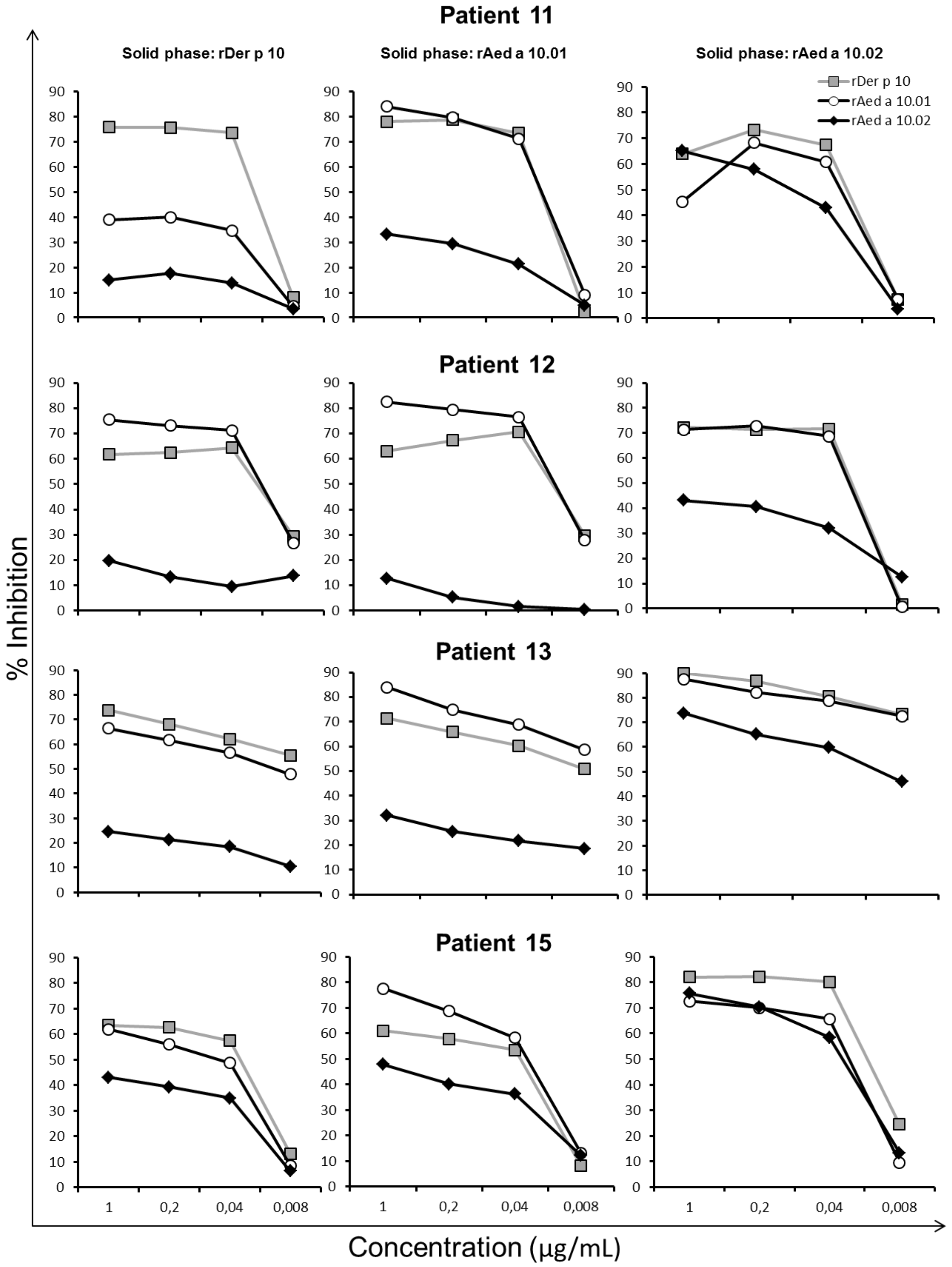


Figure 1. ELISA inhibition assays. Sera from 4 HDM allergic individuals were adsorbed with different concentrations of rDer p 10, rAed a 10.0101 and rAed a 10.0201, and assays for IgE-reactivity against the three tropomyosins

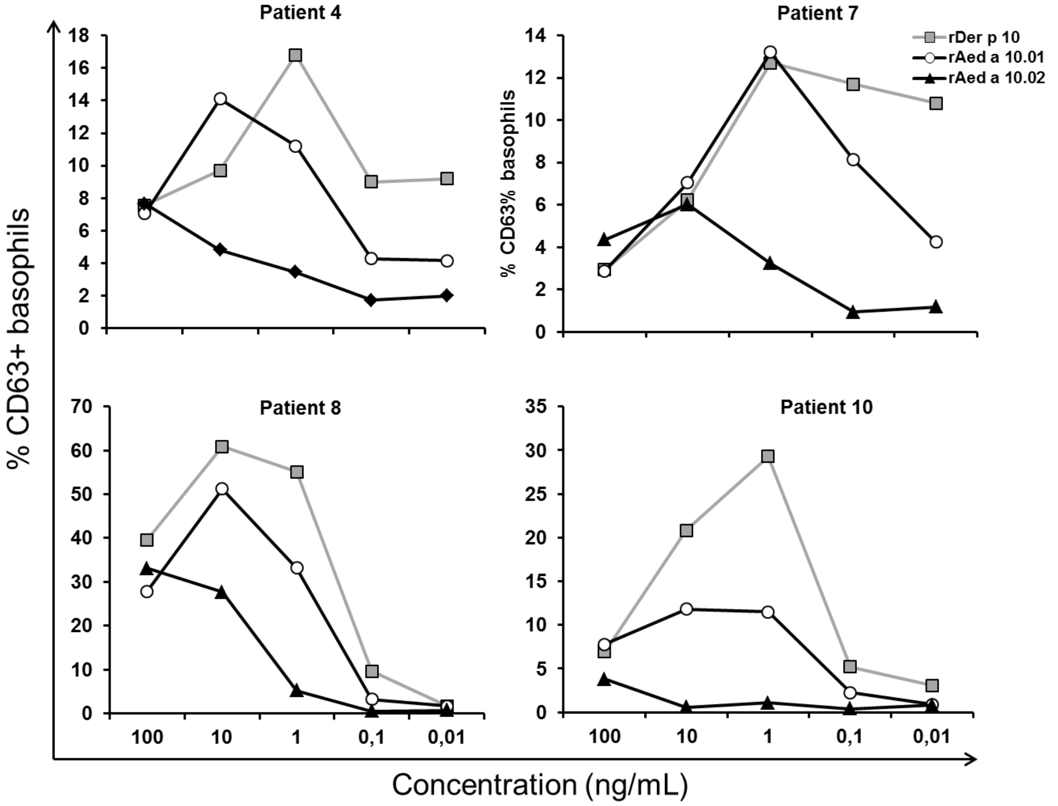


Figure 2. Basophil activation test. Stripped basophils sensitized with the IgE from the sera of 4 HDM-allergic patients were stimulated with different concentrations of rDer p 10, rAed a 10.0101 and rAed a 10.0201. Activation of basophils was expressed as the percentage of CD63$^+$ basophils.

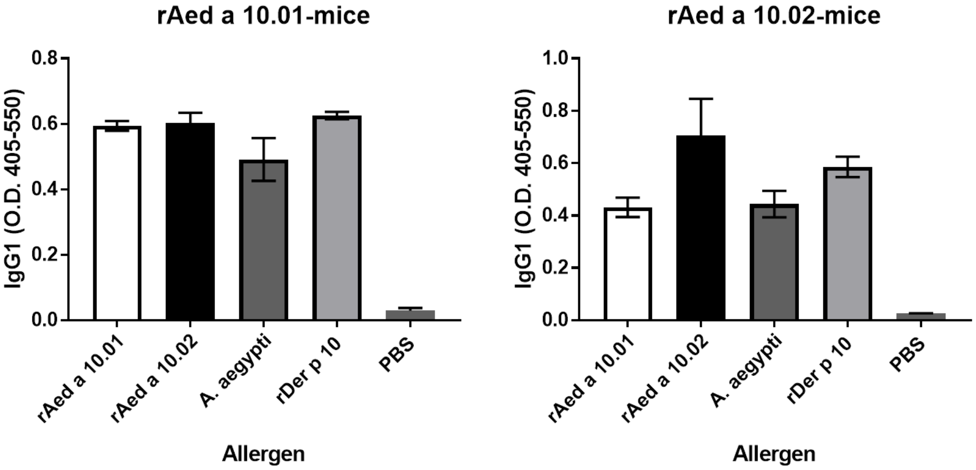


Figure 3. Cross-reactivity among tropomyosins with murine IgG1. Sera from rAed a 10.0101-mice and rAed a 10.0201-mice was tested for specific IgG1 against tropomyosins from *D. pteronyssinus* and *A. aegypti.*

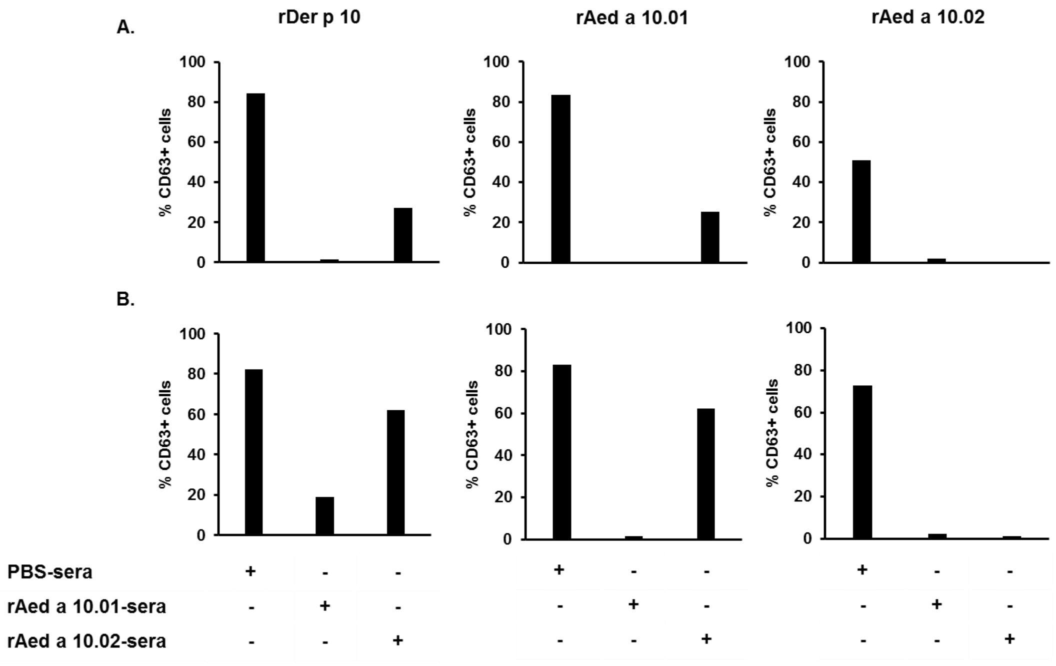


Figure 4. Inhibition of the BAT assay. Allergens at 10 µg/mL and 100 µg/mL were incubated with the sera from rAed a 10.0101-mice and rAed 10.0201-mice. BAT assay was performed as described.

RESULTS

Characterization of folded biologically active rAed a 10.01 and Aed a 10.02

The analysis of the amino acid sequence alignment revealed that Der p 10 shares 78.9% of identity with Aed a 10.01 and 62.7% with Aed a 10.02. To evaluate if these allergens also shared a similar secondary structure, the far-UV CD spectra of rDer p 10, rAed a 10.01 and rAed a 10.02 were recorded at room temperature. All spectra showed a maximum at about 191 nm and two minima at about 208 nm and 222 nm (Online repository). These results indicated that rAed a 10.01 and rAed a 10.02 are folded and present an α-helical structure, similar to rDer p 10.

IgE cross-reactivity of rAed a 10.01 rAed a 10.02 and rDer p 10

Fifteen HDM-allergic patients sensitized to Der p 10 showed IgE-binding to Aed a 10.01 and Aed a 10.02 in ELISA, suggesting that the 3 allergens cross-react (Table 1). We observed a tendency for a more pronounced IgE reactivity to rAed a 10.01 than to rAed a 10.02. However, no statistical differences were found.

Next, sera from patients no. 11, 12, 13 and 15 were individually pre-incubated with increasing concentrations of rDer p 10, rAed a 10.01 and rAed a 10.02 and the inhibition of IgE-binding to each tropomyosin was assessed (Fig. 1). With one exception, IgE-binding to rDer p 10 was best inhibited by rDer p 10, followed by rAed a 10.01 and rAed a 10.02. Aed a 10.01 also best inhibited IgE-binding to itself followed by rDer p 10 and rAed a 10.02. Notably, IgE-binding to solid-phase bound rAed a 10.02 was best inhibited by rDer p 10 and Aed a 10.01

rAed a 10.01 and rAed a 10.02 induce activation of basophils re-sensitized with Der p 10-specific IgE

To assess whether the cross-reactivity of Der p 10 and the mosquito-derived tropomyosins induced effector cell activation, stripped basophils were sensitized with sera from four patients containing high levels of Der p 10-specific IgE and incubated with rAed a 10.01 and rAed a 10.02, respectively (Fig. 2). rDer p 10 served as positive control. Maximum basophil activation with rDer p 10 was observed at the concentration of 1 and 100 ng/mL. In all patients, both mosquito tropomyosins induced basophil activation.

However, rAed a 10.01 induced stronger activation of basophils than rAed a 10.02.

rDer p 10 cross-reacts with murine IgG1 produced against rAed a 10.01 and rAed a 10.02

To further analyze the cross-reactivity between tropomyosin in HDM and mosquitos, we immunized mice with rAed a 10.01 and rAed a 10.02, respectively. Control mice group was immunized with PBS. Thereafter, we analysed whether the induced antibodies bound rDer p 10. Figure 3 shows that IgG1 antibodies produced against both A. aegypti-derived tropomyosins bound rDer p 10. Moreover, antibodies induced by immunization with rAed a 10.01 reacted with rAed a 10.02 and vice versa. Finally, antibodies produced against the recombinant tropomyosins reacted with their natural counterparts in the mosquito extract (Fig. 3). Next, we employed the murine sera to inhibit the activation of basophils sensitized with Der p 10-specific antibodies. rDer p 10, rAed a 10.01 and rAed a 10.02 were pre-incubated with the sera from the mice immunized with rAed a 10.01 and rAed a 10.02. The treated allergens were used in BAT assays at 10 and 100 ng/mL (Fig. 4). For the three tropomyosins, sera from rAed a 10.01-mice produced high inhibition of their capacity to activate the basophils, while sera from rAed a 10.02-mice produced lower inhibition.

rDer p 10 and A. aegypti tropomyosins cross-react at the T-cell level involving 5 amino acid regions containing T-cell epitopes.

To assess the cross-reactivity of HDM and mosquito tropomyosins at the T cell level, splenocytes from mice immunized with rAed a 10.01 and rAed a 10.02 were stimulated with different concentrations of rDer p 10 and *A. aegypti* tropomyosins. Splenocytes from rAed a 10.01-mice showed similar proliferative responses to rAed a 10.01 and rAed a 10.02 and to a lesser extent to rDer p 10 (Fig. 5). In contrast, splenocytes from rAed a 10.02-mice proliferated strongly upon stimulation with rAed a 10.01 and to a lesser extent to rAed a 10.02. These cells did not proliferate when stimulated with rDer p 10.

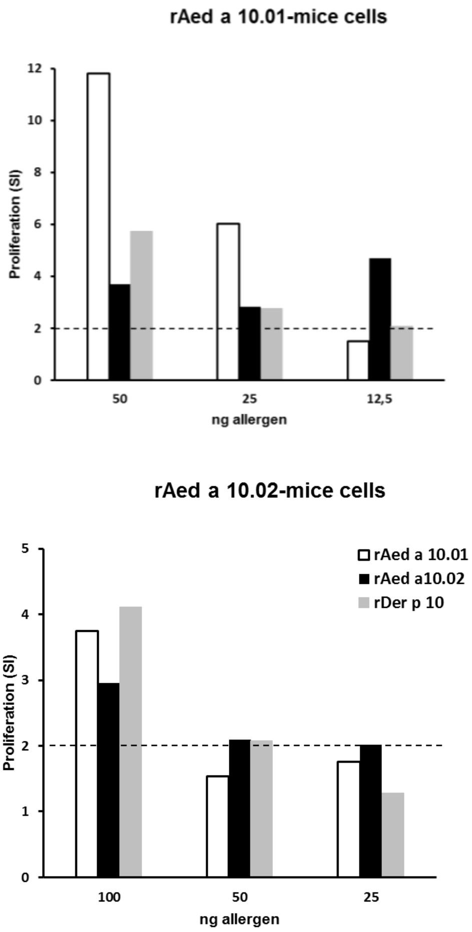
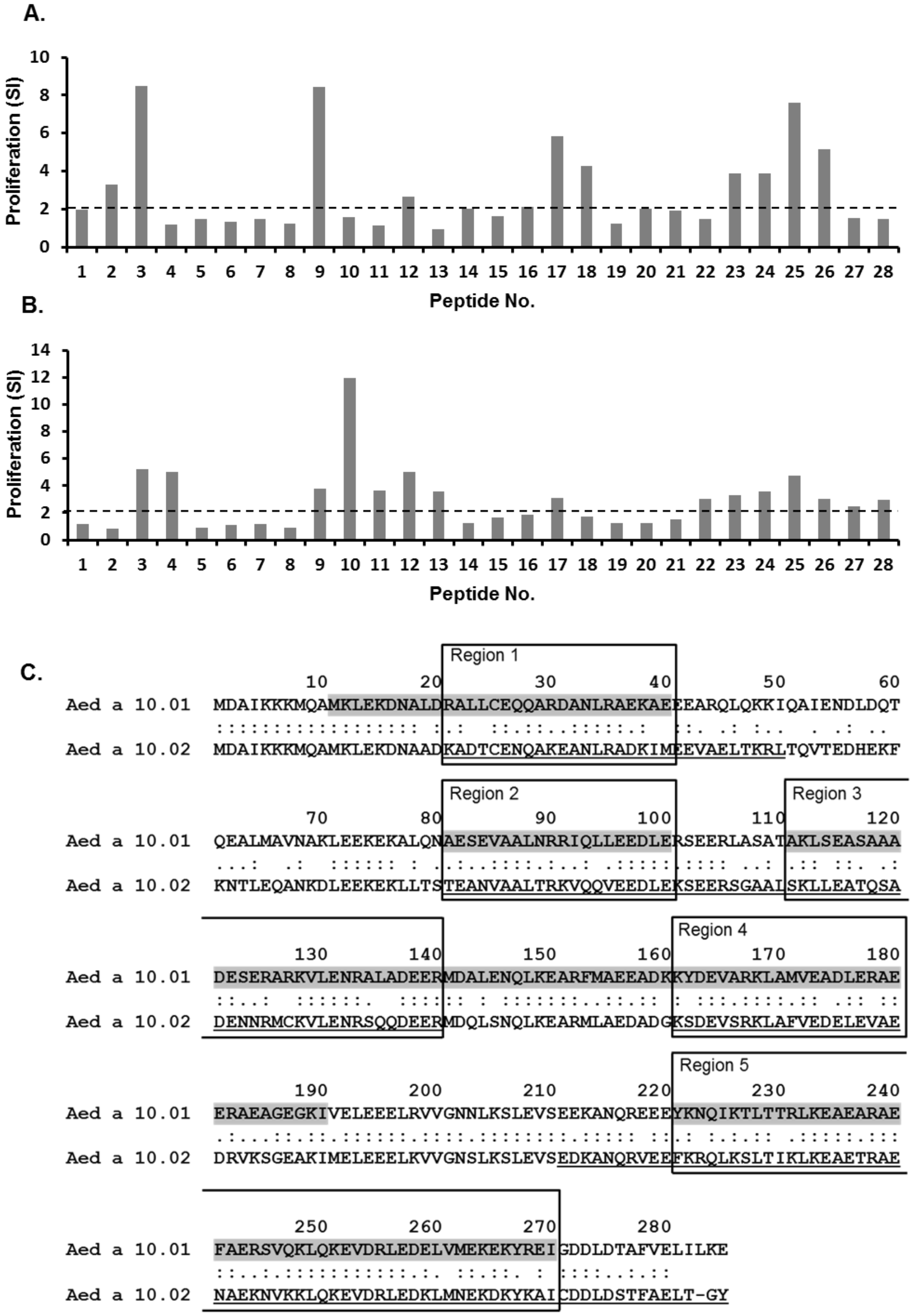


Figure 5. Lymphoproliferation assays. Splenocytes from rAed a 10.0101-mice and rAed 10.0201-mice were stimulated with different concentrations of rDer p 10, rAed a 10.0101 and rAed a 10.0201. Proliferation was measured by means of the incorporation of 3[H]-labeled thymidine.

Next, we stimulated the splenocytes with a panel of 28 overlapping peptides representing the amino acid sequence of Aed a 10.01 (Fig. 6 A and B). Peptides covering the regions Aed a 1011-40, Aed a 1081-100, Aed a 10111-190 and Aed a 10221-270 were recognized by splenocytes from Aed a 10.01-mice. Splenocytes from mice immunized with Aed a 10.02 responded to peptides covering the regions Aed a 1021-50, Aed a 1081-140, Aed a 10161-180 and Aed a 10211-285. Based on these results, five T epitopes-containing regions were detected: region 1 (amino acids 21-40), region 2 (amino acids 81-100), region 3 (amino acids 111-140), region 4 (amino acids 161-180) and region 5 (amino acids 221-270) (Fig. 6 C).



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                     10        20        30        40        50        60
Aed a 10.01  MDAIKKKMQAMKLEKDNALDRALLCEQQARDANLRAEKAEEEARQLQKKIQAIENDLDQT
             :::::::::::::::::: :.:  ::.::..:::::.:  ::. .: :..   . .: ..
Aed a 10.02  MDAIKKKMQAMKLEKDNAADKADTCENQAKEANLRADKIMEEVAELTKRLTQVTEDHEKF

                     70        80        90       100       110       120
Aed a 10.01  QEALMAVNAKLEEKEKALQNAESEVAALNRRIQLLEEDLERSEERLASATAKLSEASAAA
             ...:  .:  :::::: : ..:..::::.:..: .:::::.:::: ..: .:: ::. .:
Aed a 10.02  KNTLEQANKDLEEKEKLLTSTEANVAALTRKVQQVEEDLEKSEERSGAALSKLLEATQSA

                    130       140       150       160       170       180
Aed a 10.01  DESERARKVLENRALADEERMDALENQLKEARFMAEEADKKYDEVARKLAMVEADLERAE
             ::..:  :::::::.  :::::: : :::::::..::.:: : :::.:::::.:: .:: ::
Aed a 10.02  DENNRMCKVLENRSQQDEERMDQLSNQLKEARMLAEDADGKSDEVSRKLAFVEDELEVAE

                    190       200       210       220       230       240
Aed a 10.01  ERAEAGEGKIVELEEELRVVGNNLKSLEVSEEKANQREEEYKNQIKTLTTRLKEAEARAE
             .:...::.::.::::::.::::.::::::::.::::: ::.: :.:.:: .::::::.:::
Aed a 10.02  DRVKSGEAKIMELEEELKVVGNSLKSLEVSEDKANQRVEEFKRQLKSLTIKLKEAETRAE

                    250       260       270       280
Aed a 10.01  FAERSVQKLQKEVDRLEDELVMEKEKYREIGDDLDTAFVELILKE
             ::..:.:::::::::::::.:.  ::.::.  : ::::..:.::
Aed a 10.02  NAEKNVKKLQKEVDRLEDKLMNEKDKYKAICDDLDSTFAELT-GY
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Figure 6. T-cell epitope mapping. Splenocytes from rAed a 10.01-mice and rAed a 10.02-mice were stimulated with 20-mer peptides covering the complete sequence of Aed a 10.01. Stimulation Index ≥ 2 was considered positive.

DISCUSSION

In this study, we have analysed the humoral and cellular cross-reactivity between tropomyosins allergens from HDM (Der p 10) and two variants from A. aegypti (Aed a 10.01 and Aed a 10.02). ELISA inhibition, BAT and BAT inhibition experiments indicated that at the B-cell epitope level, Der p 10 and Aed a 10.01 cross-react and that a similar epitope spectrum is displayed by both molecules. However, lower cross-reactivity was observed between these two allergens and Aed a 10.02, indicating a lower B-cell epitope spectrum, which correlates with the lower degree of homology of Aed a 10.02 with other tropomyosins. The three tropomyosins cross-reacted with murine splenocytes and five amino acid regions containing T-epitopes seem to be involved.

The detected regions containing T-cell epitopes presented between 55 to 70% of amino acid identity and 80 to 95% of amino acid similarity when compared within Aed a 10.01, Aed a 10.02 and Der p 10. T-cell cross stimulation has been observed for epitopes with 60–80% amino acid similarity and 40–67% amino acid identity with T-cell epitopes from Bet v 1 (23). The higher homology observed in our results correlate with the high cross-reactivity within HDM and mosquito tropomyosins.

Previous experiments demonstrated the presence B-cell epitopes in tropomyosin allergens (18-20, 24, 25). Five regions highly conserved in the primary structure of several tropomyosin (18-20) are more conserved in Der p 10 and Aed a 10.01 (3), suggesting that they may be more allergenic and produce higher cross-reactivity than Aed a 10.02. This assumption was confirmed in our work by several experiments. ELISA, ELISA inhibition and BAT experiments using the sera obtained from different Austrian HDM-allergic patients with high titters of Der p 10 specific-IgE showed that the IgE-binding and inhibition capacity achieved with Der p 10 and Aed a 10.01 was higher than with Aed a 10.02 (Table 1 and Fig. 1 and 2).

Interestingly, BAT inhibition experiments showed that the murine anti-tropomyosin sera from Aed a 10.01-mice contained antibodies that highly inhibited the activation of basophils induced by the tropomyosins. However, Aed a 10.02-mice sera produced lower inhibition (Fig. 4). Since the inhibitory capacity of the mice sera may be explained by the presence of IgG antibodies, we hypothesize that IgE and IgG B-cell epitopes overlap in the 3-dimensional structure and that these epitopes are more represented in the Aed a 10.01. This discovery may have clinical implications, since it suggests that the use of tropomyosin in immunotherapy strategies may result in the induction of blocking antibodies.

Wai, C.Y.Y. et al. (26) Identified six peptides derived from the shrimp tropomyosin, Met e 1, which contained T-cell epitopes recognized by mice. These peptides showed regulatory capacity of the allergic response in a mouse model of allergy to Met e 1, which involved the production of blocking IgG2a. The six regions identified were: T1 (26-45), T2 (56-75), T3 (86-105), T4 (146-165), T5 (221-240) and T6 (251-270). When compared with the regions identified in our study we found that T1, T2 and T3 partially coincide with regions 1, 2 and 3 described by us, and that T4, T5 and T6 are mostly contained in region 4 and 5 where the sequence identity and similarity between Aed a 10.01 and Met e 1 is higher than 70% and 80%, respectively. These findings indicate that the regions T4, T5 and T6, and to a lesser extent T1, T2 and T3 described by Wai, C.Y.Y. et al, and the regions described in our study, may involve the same T-cell epitopes. Although we didn’t evaluate the regulatory role of our peptides, we hypothesize that the IgG response and the BAT blocking capacity of the mice sera are indicative of a potential capacity to induce protective response in a similar way as Wai, C.Y.Y. et al. reported.

Furthermore, we believe that using a tropomyosin allergen in an immunotherapy approach may result in the induction of protection against a broad spectrum of tropomyosins from different species, which is explained by the presence of cross-reactive T-cell epitopes. Some studies support this hypothesis. For instance, sublingual immunotherapy for house dust mites resulted in an improvement of shrimp allergy (27) and subcutaneous immunotherapy has also been reported to be associated with a decrease in specific serum IgE and resolution of shrimp and squid allergy (28).

The clinical significance of cross-reactivity relies on different aspects. Patients sensitized to a primary sensitizing allergen may develop allergic symptoms upon exposure to a cross-reactive homologue molecule containing cross-reactive B-cell epitopes (29, 30). Our experimental data using specific IgE from HDM allergic patients, sensitized to Der p 10, demonstrated that Der p 10 and A. aegypti tropomyosins cross-react at the B-cell epitope level, from a molecular and functional point of view. Since the mosquito species A. aegypti is not present in Austria, we assume that D. pteronyssinus is the primary sensitizer and exposure to cross-reactive mosquito tropomyosin may lead to allergic symptoms through D. pteronyssinus-specific IgE. Another possibility is that the presence of cross-reactive T-cell epitopes may result in the clonal expansion of the T lymphocytes elicited by the primary sensitizer; leading to an exacerbation of the immune response and the allergic symptoms (10, 31, 32). Accordingly, the presence of cross-reactive T and B-epitopes shared in the structure of the tropomyosins analysed indicates that both phenomena may occur between HDM and mosquito tropomyosins.

We used BALB/c mice for the cellular cross-reactivity studies and T-cell epitope mapping. This may not mimic the natural sensitization process in humans, since different HLA molecules and different T-cell epitopes may be involved. However, Ravkov, E. et al. (21) reported 17 T-cell epitopes from shrimp tropomyosin restricted to multiple MHC class II alleles. We observed that 9 peptides reported by Ravkov, E. et al. significantly coincide with regions 1, 3, 4 and 5 from our study, suggesting that the T-cell epitopes recognized by the immunized mice may be the same that the human population recognizes through different HLA classes. Although we are referring to allergens from different species, the high homology among tropomyosins from shrimp and mosquito support this conclusion.

In conclusion, we have demonstrated that tropomyosins from HDM and A. aegypti display humoral and T-cell cross-reactivity involving five potential T cell-activating regions. Different degrees of humoral cross-reactivity were observed depending on the degree of homology. As previously suggested, a higher homology resulted in higher degree of cross-reactivity. It seems that this cross-reactivity occurs independently from a natural exposure to these allergens. Our results may have important clinical impacts which should be further analysed.

Conflict of Interest: none.

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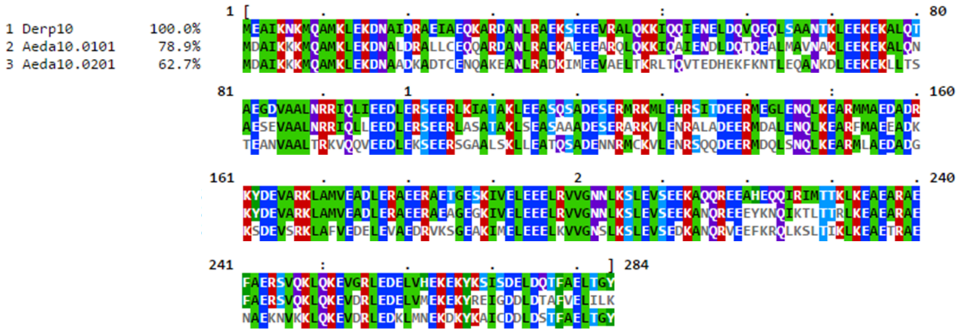
A.



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                                 1 [       .         .         .         .         :         .         .         . 80
1 Derp10         100.0%            MEAIKNKMQAMKLEKDNAIDRAEIAEQKARDANLRAEKSEEEVRALQKKIQQIENELDQVQEQLSAANTKLEEKEKALQT
2 Aeda10.0101     78.9%            MDAIKKKMQAMKLEKDNALDRALLCEQQARDANLRAEKAEEEARQLQKKIQAIENDLDQTQEALMAVNAKLEEKEKALQN
3 Aeda10.0201     62.7%            MDAIKKKMQAMKLEKDNAADKADTCENQAKEANLRADKIMEEVAELTKRLTQVTEDHEKFKNTLEQANKDLEEKEKLLTS

                                81         .         1         .         .         .         .         :         . 160
                                   AEGDVAALNRRIQLIEEDLERSEERLKIATAKLEEASQSADESERMRKMLEHRSITDEERMEGLENQLKEARMMAEDADR
                                   AESEVAALNRRIQLLEEDLERSEERLASATAKLSEASAAADESERARKVLENRALADEERMDALENQLKEARFMAEEADK
                                   TEANVAALTRKVQQVEEDLEKSEERSGAALSKLLEATQSADENNRMCKVLENRSQQDEERMDQLSNQLKEARMLAEDADG

                               161         .         .         .         2         .         .         .         . 240
                                   KYDEVARKLAMVEADLERAEERAETGESKIVELEEELRVVGNNLKSLEVSEEKAQQREEAHEQQIRIMTTKLKEAEARAE
                                   KYDEVARKLAMVEADLERAEERAEAGEGKIVELEEELRVVGNNLKSLEVSEEKANQREEEYKNQIKTLTTRLKEAEARAE
                                   KSDEVSRKLAFVEDELEVAEDRVKSGEAKIMELEEELKVVGNSLKSLEVSEDKANQRVEEFKRQLKSLTIKLKEAETRAE

                               241         :         .         .         .   ] 284
                                   FAERSVQKLQKEVGRLEDELVHEKEKYKSISDELDQTFAELTGY
                                   FAERSVQKLQKEVDRLEDELVMEKEKYREIGDDLDTAFVELILK
                                   NAEKNVKKLQKEVDRLEDKLMNEKDKYKAICDDLDSTFAELTGY
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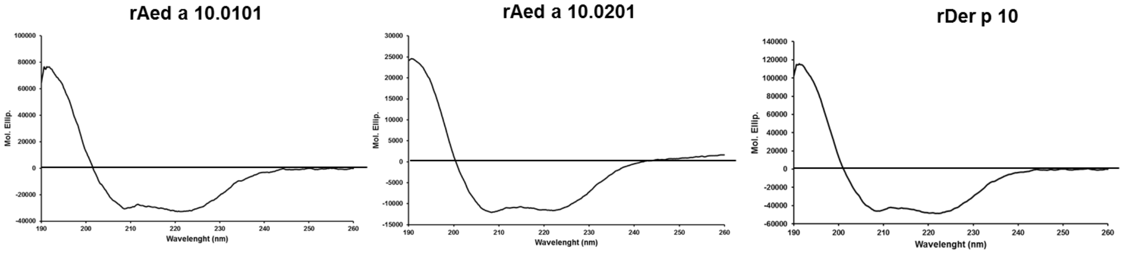
B.



Online repository. Multiple sequence alignments and circular dichroisms of rDer p 10, rAed a 10.0101 and rAed a 10.0201. Comparison of the amino acid sequences was performed with webPRANK. CD showed folded tropomyosins.

VII. GLOBAL DISCUSSION OF THE RESULTS

A. *A. aegypti* as a source of allergens

Allergy diseases have become a global health problem that affects the lifestyle of the population and, in some cases, puts the life of the individuals at risk. Several sources of allergens have been well characterized and many IgE-binding components have been identified, purified and registered in the allergen databases. Despite these efforts, and the advances made in the field of Allergology, several allergen sources remain to be identified and further studied. As we previously explained, we had reasons to believe that mosquitoes, including the species *A. aegypti,* are a source of important allergens to which the individuals may be exposed during the mosquito bite, by inhalation, or further contact. As a result of this exposure, an allergic response may be induced in the atopic individual, or may exacerbate the allergic response previously initiated against a related, or non-related species, such as mites, cockroaches and shrimps.

The WHO/IUIS nomenclature system provides general requirements to consider a protein as an allergen. These molecules must satisfy biochemical criteria that define the molecular structure of the proteins, and immunological criteria, which define their importance as an allergen (159). The biochemical criteria should provide indications, whereby other investigators can identify the same allergen and make comparative studies. These criteria are based on establishing protein purity and physicochemical properties (160). In general, having the protein sequence available is one of the most important requirements. The immunological criteria include demonstrating that the purified allergen has allergenic activity, both *in vitro* and *in vivo* by IgE-binding assays and mediator release experiments. Preferably, at least 50 sera should be screened to determine the prevalence of IgE reactivity, and allergens can be included in the nomenclature if the prevalence is >5% and if they elicit IgE response in as few as 5 patients.

Considering these criteria, our results demonstrate that the mosquito *A. aegypti* is a source of allergens. We have reported several IgE-binding proteins with their physicochemical characteristics including molecular weights, isoelectric points and partial sequences, and their capacity to bind the IgE from allergic individuals and cross-react with similar allergens. For the mosquito extracts, and some recombinant allergens (rAed a 8 and rAed a 10), their capacity to induce the activation of basophils was also demonstrated. In the case of rAed a 10 variants and rAed a 8, we demonstrated that they conserve their capacity to induce lymphoproliferation. Furthermore, T-cell cross-reactive epitopes from *A. aegypti* tropomyosin were identified. Altogether, these discoveries demonstrate that *A. aegypti* allergens have the molecular and immunological requirements to induce an immune response leading to the development of allergic symptoms.

Although our experimental data demonstrate that *A. aegypti* is a source of allergens, in real life, the onset of the allergic response requires that the atopic individual is exposed to these molecules. The presence of mosquito material in the air and settled house dust has been demonstrated. However, further studies directed to demonstrate the correlation between allergen exposure and the allergic response are needed. We have made some advances in this topic, demonstrating that more than 50% of the allergic patients from the Municipality of Medellin, Colombia, suffering from allergic rhinitis, have positive SPT to a whole body *A. aegypti* extract (See attachment 1). Measuring the presence of mosquito allergens in house dust and in the air is important to determine whether mosquito exposure is a risk factor for respiratory allergies, as has been demonstrated for mites, cockroaches and other insects.

B. Identification of IgE-binding proteins and their relationship with allergic diseases

The first step that we followed to study whether *A. aegypti* is a source of allergens was the identification of IgE-binding proteins. Currently, there are different approaches to achieve this task. One of them is the purification of the natural molecule from the source, which usually is time consuming and requires the availability of large amounts of raw material. However, it is advantageous because it guarantees the use of the natural allergen. We used this approach to identify and purify native tropomyosin from *A. aegypti* in a two steps protocol involving size exclusion and ionic exchange chromatography. The purified tropomyosin consisted of a mixture of several variants or isoforms. The most abundant were Aed a 10.0101 and Aed a10.0201. This task was possible, in part, because tropomyosin is abundant in the mosquito extract. The purification of tropomyosin follows a strategy involving ammonium sulphate precipitation and isoelectric precipitation (161). However, in the case of *A. aegypti* the presence of several proteins that remain bound to tropomyosin (mainly myosin) is a problem that can be solved by direct elution from the electrophoresed gel. Other researchers have combined partial purification steps, such as acetone precipitation and boiling, followed by ionic exchange chromatography (162, 163).

The use of proteomic tools has become one of the preferred options to identify allergens because it allows the analysis of the whole spectrum of proteins expressed by an organism. The identified molecules have already undergone the expression and translational modifications experienced naturally. Nowadays, as a result of the application of proteomic in the discovery of novel allergens, the field of allergy research has included in the “omic” era the concept of “Allergenome” (151, 164-166).

Within this project, we successfully identified allergens from *A. aegypti* in a large scale. A total of 10 different IgE-binding proteins, some of them including variants, or isoforms, were identified. Furthermore, five novel allergens were deposited in the allergen database.

One intriguing question about mosquito allergy consists in determining the role that their allergens play in the different manifestations of mosquito allergy. Several studies have established that saliva-derived allergens, which are injected into the skin during the mosquito bite, induce cutaneous allergic reactions. Four allergens, Aed a 1, Aed a 2, Aed a 3 and Aed a 4, are involved in this cutaneous reactions (62, 106, 109, 167). As a result of our research, five novel *A. aegypti* allergens were reported and several IgE-binding proteins identified. None of the allergens that we identified correspond to the previously reported salivary-allergens. The difference may be due to the different populations used in the IgE-binding screenings and the source of the raw materials used. We used the sera from patients residing in tropical regions and suffering respiratory allergies. In other studies, sera from patients residing at different latitudes and suffering cutaneous allergic responses were used to study the relevance of mosquito allergens. These differences strongly suggest that mosquito allergens may play different roles in allergic diseases depending on the location of the mosquito and the route of exposure. In tropical regions, and other latitudes where mosquitoes are abundant, the individuals may be exposed to their antigens by different routes of exposure and experience cutaneous as well as other clinical manifestations of allergic diseases. Within the group of allergens that we identified, several correspond to homologues of allergens from other sources, like tropomyosin (168, 169), heat shock cognate-70 (151), cyclophilins (170-172), among others, which further suggests that mosquito body-derived proteins may play a role as allergens.

The results from the study of the Allergenome of *A. aegypti* allowed advances in the component resolved diagnosis of mosquito allergy. Although a variable pattern of sensitization was observed in a group of 15 allergic individuals from the Caribbean Island of Martinique, we observed that a mixture of three allergens (Aed a 6, Aed a 8 and Aed a 10) may be enough to identify more than 80% of *A. aegypti* allergic individuals. This result has a considerable impact because it indicates that a diagnosis tool based on the use of a reduced number of mosquito allergens may successfully identify mosquito allergic individuals.

C. Is *A. aegypti* a primary sensitizer?

An open question is whether mosquito allergens represent a group of allergens with the capacity to induce an immune response leading to the appearance of allergic symptoms, or whether the IgE-binding capacity of some of these proteins is the result of a cross-reactivity process.

From an experimental point of view, a way to define whether an allergen source is the primary sensitizer involves the demonstration that some patients are mono-sensitized to their allergens and the results of competition, or inhibition assays which show that the response is of a higher affinity (173, 174). We used two populations of patients for the different studies. One population of 15 house dust mite allergic patients from Austria sensitized to Der p 10 (tropomyosin). All the patients showed cross-sensitization to *A. aegypti* tropomyosins (rAed a 10.0101 and rAed a 10.0201) at the molecular and functional level. However, inhibition experiments using 4 individual sera showed that Der p 10 produced higher inhibition of the IgE-binding against itself and the mosquito tropomyosins. These results are in agreement with the fact that in Austria there is no *A. aegypti*, suggesting that mite allergens are the primary sensitizers which cross-react with mosquito proteins. In another study analyzing a population from the Caribbean Island of Martinique, where mites and *A. aegypti* cohabit, the results of competition assays with whole extracts showed that *D. pteronyssinus, B. tropicalis* and *A. aegypti* extracts produce similar inhibition of the IgE-binding. However, in almost all the patients, the levels of serum IgE was higher against mites than the levels against mosquito. In this population 3 out of 34 patients showed specific IgE to *A. aegypti* alone, and three co-sensitization to *A. aegypti* and *P. americana*.

Altogether, these results suggest that in some cases, *A. aegypti* may act as a primary sensitizer and that *A. aegypti* allergens strongly cross-react with arthropod-derived allergens. In areas where these species cohabit, independent sensitization to each of them may occur. More data and studies in other communities are needed to definitively answer this question, since exposure to mites and mosquitoes varies widely. Epidemiological studies are warranted.

D. Recombinant *A. aegypti* allergens and the contribution to the study of mosquito allergens

A limiting factor for the study of allergens is the low availability and difficulties to obtain purified molecules. In the case of mosquitoes, this is evident, since neither native proteins, nor allergens, have been obtained as purified molecules. The scientific community, in addition, has been interested in finding new therapeutic tools to help in the improvement of the diagnosis and immunotherapy of allergic diseases. Molecular cloning has provided a practical and efficient tool to obtain highly purified molecules for these purposes. The era of molecular cloning of mite allergenic molecules was initiated in 1980s with the report of a cDNA clone coding for the allergen Der p 1 (175, 176). Since that time, many recombinant allergens from variable sources have been obtained as recombinant proteins, accelerating the comprehension of the role of these molecules in the mechanisms and pathophysiology of allergic diseases. Furthermore, the relative easiness with which these molecules can be produced and modified has helped to design improved approaches for the diagnosis and therapy of these diseases.

Recombinant allergens can replace native allergens if they conserve similar folding and biochemical properties as the natural protein. In this study, three recombinant mosquito allergens where expressed in *E. coli* and purified. Two variants of tropomyosin (rAed a 10.0101 and rAed a 10.0201) and rAed a 8 were obtained. The three allergens showed a similar capacity to bind IgE and IgG antibodies, to activate basophils, to induce the immunization of mice and to induce lymphoproliferation as compared with the native allergens. The tropomyosin variants showed an α-helical folding similar to Der p 10. Altogether, these results demonstrate that the three allergens may be used in replacement of the natural proteins for allergy research, diagnosis and treatment. The study of these molecules helped to establish that the mosquito allergens may also induce the initiation of an immune response and activate the mechanisms that lead to the release of mediators of the allergic response. We have also further investigated the importance of *A. aegypti* tropomyosins in mosquito sensitized patients and studied its molecular and functional cross-reactivity.

We demonstrated that Aed a 8 is an important allergen recognized by more than 40% of the mosquito allergic population. An important discovery related to the immune response induced by rAed a 8 in mice was that high levels of IgG antibodies were produced, especially of the subclass IgG2a which has been suggested as a marker of a successful specific immunotherapy and as a blocking antibody that inhibit the interaction of the IgE with the allergens in mouse models of allergy (177, 178). These results are similar to those obtained in other studies showing that mosquito allergens, including heat shock protein-70, may induce a protective immune response that potentially protects against the allergic response (77, 179). The potential regulatory effect of Aed a 8 must be further studied and considered in the design of new approaches of immunotherapy for mosquito allergies.

E. Allergenic cross-reactivity among *A. aegypti* and other arthropods: The role of mosquito tropomyosins

According to the evolutionary relationship and the demonstration of the presence of shared allergens (some of them pan-allergens) among *A. aegypti* and other arthropods, it was expected to detect allergenic cross-reactivity. Our results with tropomyosins from *A. aegypti* and the identification of a large set of cross-reactive allergens from this mosquito, confirmed this hypothesis. However, we further studied this phenomenon at the molecular and functional levels, and reported a group of potentially cross-reactive allergens.

Cross-reactivity between groups of allergens may occur with different antibody classes. We initially focused on IgE given its direct involvement in Th2 and allergic responses. Using ELISA, immunoblotting and inhibition assays, we found that *A. aegypti* strongly cross-react with shrimp (*L. vannamei*), followed by mites (*D. pteronyssinus* followed by *B. tropicalis*) and cockroach (*P. americana)*. The observed cross-reactivity involved at least 11 bands identified in a 1-dimensional immunoblotting. We further characterized five of these bands: Odorant Binding Protein, Mitochondrial cytochrome C, Cyclophilin (PPIase), an unknown protein “AAEL001668-PA” and tropomyosin. Interestingly, tropomyosin and cyclophilins are protein families which are highly conserved in their primary and tertiary structure among several species from different Phylum and considered pan-allergens (180-182). The 3 other proteins are also highly conserved in different species and represent potential pan-allergens.

For the identification and characterization of these cross-reactive molecules, we used a set of more than 30 sera from allergic patients from Martinique. As mentioned previously, this island has a tropical climate and house dust mites, cockroaches, mosquitoes and other insects are abundant. They represent the main risk factors for allergic sensitization and allergic diseases. Sensitization to shrimps and crabs is also very frequent (183, 184). In this population, sensitization to *A. aegypti* was 64.7% among allergic individuals. This prevalence is one of the highest ever reported (122, 185, 186) and similar to the prevalence of sensitizations to mites in allergic individuals. This result, together with the high cross-reactivity observed, indicates that mosquitoes are environmental factors that should be considered in health programs for prevention and protocols of diagnosis and treatment of allergic diseases in tropical areas.

Cross-reactivity involving IgE-binding epitopes indicates that the allergic response elicited against a primary sensitizer may be stimulated by exposure to an allergen containing the same, or similar epitopes in its structure, resulting in the appearance of the allergic responses (80, 81). It is necessary that cross-reactivity between two or more molecules occurs by recognition of similar IgE-binding epitopes and cross-activation of effector cells. However, in some cases IgE-cross binding may not lead to an allergic response in the exposed patient and is limited to a simple reactivity with no clinical consequences.

The cross-reactivity phenomenon among allergens has immunological and clinical consequences that go beyond IgE-binding. In addition to the IgE-binding epitopes, IgG-binding epitopes may also participate in the cross-reactivity phenomenon. In this case, the production of IgG subclasses throughout a desensitization process against a particular allergen may lead to the decrease or disappearance of this response to a different, but cross-reactive molecule (177).

We analyzed the cross-reactivity between tropomyosins from mite (Der p 10) and *A. aegypti* (Aed a 10.0101 and Aed a 10.0201). By IgE-binding assays, basophil activation and inhibition experiments, we confirmed that Der p 10 and both tropomyosins cross-react, and that the degree of cross-reactivity correlated with the degree of homology between these allergens. Cross-reactivity between these tropomyosins occurs with human and murine IgE and murine IgG and the results suggest that the IgG and IgE-binding epitopes are co-localized on the structure of the allergen. These results suggest that the antibody cross-reactivity between tropomyosins from *A. aegypti* and mites may lead to the activation of the allergic cascade, independently of which molecule is the primary sensitizer. This phenomenon occurs because these molecules share B-cell epitopes that bind to the IgE on the surface of effector cells, leading to the IgE cross-linking and activation and degranulation.

Cross-reactivity may also occur at the level of T-cells, involving T-cell epitopes that may, or may not, correspond to the IgE-binding epitopes. Furthermore, a protein that is neither a primary sensitizer, nor an allergen, may have T-cell cross-reactive epitopes. From this assumption, some immunological and clinical implications may be postulated as follows (82, 187). Cross-reactive T cells elicited by allergen exposure will: (1) be selectively expanded and boosted by exposure to additional allergens containing the conserved epitope and (2) generate help for any B cell specific for an allergen cross-reactive at the T-cell level through a classic antigen bridge–linked T-cell/B-cell help mechanism, regardless of whether the IgE response is cross-reactive.

Using murine splenocytes, we found that there is T-cell cross-reactivity among mite and mosquito tropomyosins and that this phenomenon involves five regions containing T-cell epitopes. The presence of these epitopes may lead to the boost and expansion of T cells and generate help to B cells.

VIII. CONCLUSIONS

1. The mosquito species *A. aegypti* is an important source of allergens that may play a role in the pathogenesis of allergic diseases, especially in tropical and subtropical areas, where the allergic population may be monosensitized, or polysensitized to *A. aegypti* and other arthropods.
2. Two groups of different allergens seem to play independent roles in the different manifestations of mosquito induced allergic diseases, i.e., salivary and somatic antigens.
3. More than 10 allergens are involved in mosquito allergy.
4. These allergens may potentially participate in the activation of the allergic cascade because they present the B-cell and T-cell epitopes needed for the stimulation of the lymphocytes and effector cells that mediate the emergence of the inflammatory symptoms.
5. The mixture of three mosquito allergens may be enough to identify more than 80% of the mosquito sensitized population.
6. Some of the *A. aegypti* allergens cross-react with other arthropod-derived allergens at the molecular and functional level.
7. The studies with *A. aegypti* tropomyosin revealed that cross-reactivity may occur with IgE and IgG subclasses and that IgE-cross binding may lead to cross-activation of effector cells like basophils.
8. These cross-reactive allergens share T-cell cross-reactive epitopes that may boost the immune response generated by the primary sensitizer.
9. Mosquito allergens may be produced as recombinant molecules that mimic the natural allergens in their structural and immunological properties.
10. Aed a 8 is a potential major allergen that should be further studied.
11. Studies of the immune response elicited by rAed a 8 in mice indicated that this allergen may have an intrinsic immunoregulatory role.

IX. CLAIMS

The impact that this project represent for the scientific and medical community are manifested in the following claims, which are reported for the first time:

1. The Allergenome of *A. aegypti* was studied by the application of proteomic tools, revealing 25 IgE-binding spots that corresponded to 10 different proteins.

2. The native tropomyosin from *A. aegypti* was purified and its antibody-binding capacity studied.

3. Three biologically active recombinant allergens from the *A. aegypti* body (rAed a 10.0101, rAed a 10.0201 and rAed a 8), were expressed in *E. coli.*

4. The IgE-cross reactivity among *A. aegypti* and mites, shrimp and cockroach, together with the molecular components involved were studied and reported.

5. Five amino acid regions from the sequence of Aed a 10.0101 that contains T-cell cross-reactive epitopes were reported.

6. Six novel *A. aegypti* allergens were deposited in the database of the WHO/IUIS Allergen Nomenclature Sub-committee (www.allergen.org): Aed a 5 (Sarcoplasmic Ca+ (EF-hand) binding protein), Aed a 6 (Porin 3), Aed a 7 (Un-defined protein), Aed a 8 (Heat shock cognate-70), Aed a 10 (Two variants of tropomyosin, Aed a 10.0101 and Aed a 10.0201) and Aed a 11 (Lysosomal aspartic protease).

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respiratory allergy to mite and a food allergy to crab and shrimp. *Eur Ann Allergy Clin Immunol* 2007;**39**(3):85-88.

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XI. ATTACHMENTS

Attachment 1.

Abstract accepted for "Poster Presentation" at The European Academy of Allergy & Clinical Immunology (EAACI) Annual Meeting, Helsinki, Finland. 17-21 June 2017.

Clinical Impact of mosquito *Aedes aegypti* in allergic respiratory diseases.

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1. Group of Experimental and Clinical Allergy, IPS Universitaria, University of Antioquia. Medellín, Colombia.
2. Institute for Immunological Research, University of Cartagena. Cartagena, Colombia.
3. Foundation for the Development of Medical and Biological Sciences (FUNDEMEB). Cartagena, Colombia.

BACKGROUND: Allergic reactions to *Aedes aegypti* are associated with saliva allergens introduced in the skin by mosquito bites. However, several allergens have been described in the body of this insect, suggesting that they may participation in the induction of allergic respiratory diseases.

OBJECTIVE: To investigate the clinical relevance of the *A. aegypti* mosquito in allergic rhinitis.

METHODS: Skin Prick Test (SPT) with whole body extracts of *A. aegypti*, *Blomia tropicalis* and *Dermatophagoides* spp., were performed in 29 patients with rhinitis and 12 healthy subjects (controls) residing in Medellín, a tropical city in Colombia.

All subjects signed informed consent forms for this study and were challenged with either 200 μL of saline, or 200 μL of an extract of *A. aegypti* (Inmunotek SL, Madrid, Spain) containing 1 mg of protein/ml and diluted in the same saline solution. Each patient received a final concentration of 200 μg of protein per challenge. In addition, serum IgE, IgG and IgG4 levels to *A. aegypti* whole body extract was determined by ELISA.

RESULTS: Eighteen (18) patients with rhinitis (62%) were SPT positive to the *A. aegypti* extract. All subjects in the rhinitis group except 2 (27 individuals; 93.1%) were SPT positive to mite extracts. One control subject showed positive SPT to *A. aegypti*. Ten of eighteen (55.5%) allergic patients with positive SPT to *A. aegypti* were positive by nasal challenge test (see Figure 1). Two patients with a positive nasal challenge and SPT to mosquito extract were SPT negative to mites. None of the individuals tested were positive with the saline challenge test. In the group of allergic patients (n = 29), the frequency of positive IgE, IgG and IgG4 levels to *A. aegypti* was 27.5, 69.0 and 28.0 %, respectively.

CONCLUSION: *A. aegypti* can induce signs and symptoms of allergic rhinitis in patients with allergic rhinitis in a tropical environment. Whether, or not sensitization to *A. aegypti* is induced by cross-reactivity with other allergenic sources, such as

mites and cockroaches, the positive nasal provocation test indicates that IgE sensitization to *A. aegypti* may be clinically relevant and could be induced not only by mosquito bites but also by the inhalation of their allergens. The potential allergenic role of each *A. aegypti* protein should be further investigated.

Summary of the results.

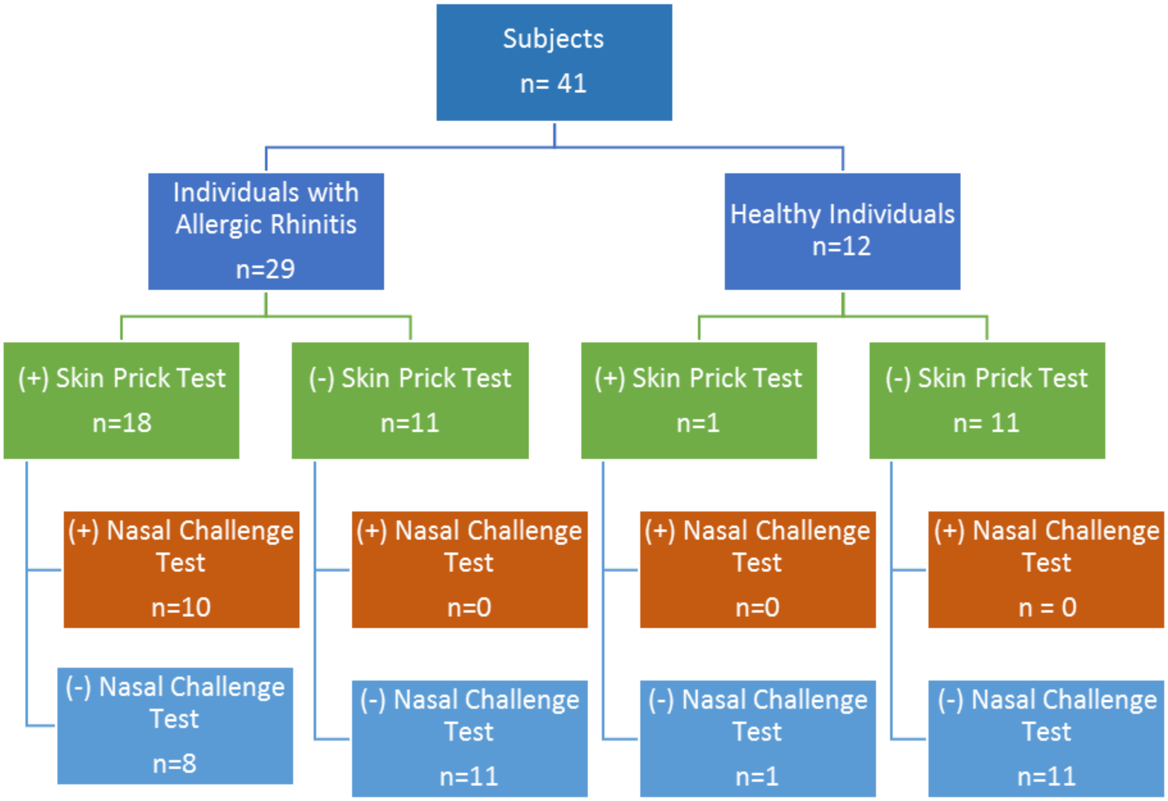

Figure 1. Description of the patient population and the main results.

Attachment 2.

CURRICULUM VITAE

JOSÉ FERNANDO CANTILLO MERCADO, PhD.

Cantillo, J.F.

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Place & Date of Birth: Cartagena de Indias (Colombia), May 10, 1985
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EDUCATION

PhD. IN BIOCHEMISTRY, MOLECULAR BIOLOGY AND BIOMEDICINE
Complutense University of Madrid, Madrid, Spain. 2012 - 2017.
Thesis Title: Identification, cloning, purification and immunological characterization of unique and cross-reactive allergens of the mosquito species Aedes aegypti.
PhD Thesis Directors: Dr. Enrique Fernández-Caldas PhD. and Dr. Leonardo Puerta PhD.

MASTER IN IMMUNOLOGY
University of Cartagena, Cartagena de Indias, Colombia. 2009 - 2010.
Project Title: Purification and IgE reactivity of a chimeric protein containing epitopes from different allergens of the mite Dermatophagoides pteronyssinus
Project Supervisor: Dr. Leonardo Puerta PhD.

BACHELOR'S DEGREE IN PHARMACEUTICAL CHEMIST
University of Cartagena, Cartagena de Indias, Colombia. 2003 - 2008.
Final Degree Project Title: Determination of the acute toxicity, hemolytic, antioxidant and antimicrobial activities of extracts of Justicia secunda (Vahl).
Project Supervisor: Rosa Baldiris MSc.

HONORS & AWARDS

2016: Best Abstract Prize at the EAACI Congress 2016 of the European Academy of Allergy and Clinical Immunology in Vienna, Austria, 11 – 15 June.

2016: Best Poster Prize at the Junior Member and Affiliates (JMA) Poster Session at the EAACI Congress 2016 of the European Academy of Allergy and Clinical Immunology in Vienna, Austria, 11 – 15 June.

2016: Congress Scholarship to attend the EAACI Congress 2016. European Academy of Allergy and Clinical Immunology (EAACI).

2015: Best Poster Prize at the Junior Member and Affiliates (JMA) Poster Session at the EAACI Congress 2015 of the European Academy of Allergy and Clinical Immunology (EAACI) in Barcelona, Spain, 6 – 10 June.

2015: Research Fellowship. European Academy of Allergy and Clinical Immunology (EAACI).

2014: Congress Scholarship to attend the EAACI Congress 2014. European Academy of Allergy and Clinical Immunology (EAACI).

2013-2017: PhD Scholarship. Administrative Department of Science, Technology and Innovation of the Colombian Government (Colciencias[1]).

2010: Best poster award. National College of Pharmacists of Colombia.

2008: Young Researcher Grant. Administrative department of science, technology and innovation (Colciencias).

2008: First place: Scholarship for high academic performance during the second academic period of 2007.

2007: Second place: Scholarship for high academic performance during the second academic period of 2006.

2006: First place Scholarship for high academic performance during the second academic period of 2005.

2005: First place. Scholarship for high academic performance during the second academic period of 2004.

ACADEMIC EXPERIENCE

Professor (Cartagena de Indias, Colombia)

2011 – 2012: University Corporation Rafael Nuñez.
Cathedra of: (1) Immunology, (2) Biochemistry, (3) Genetics and (4) Biological Sciences.

Fellow in training (Vienna, Austria)

2015 – 2016: Center for Physiology and Pathophysiology. Dept. of Pathophysiology/Division of Immunopathology. Medical University of Vienna. Research project partially supported by the European Academy of Allergy and Clinical Immunology (EAACI).

PhD Student in training (Alcalá de Henares, Madrid, Spain)

2012 – 2017: Inmunotek[2]. Research and Development department. Research work for PhD thesis

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[1] Colciencias (http://www.colciencias.gov.co/)

[2] Pharmaceutical laboratory (https://www.inmunotek.com/)

CURRICULUM VITAE

JOSÉ FERNANDO CANTILLO MERCADO, PhD.

Cantillo, J.F.

Researcher (Cartagena de Indias and Bucaramanga, Colombia)
2007 – 2010: Institute for immunological research-University of Cartagena, Cartagena de Indias, Colombia. Training in techniques of Immunology research, such as IgE and IgG determinations by ELISA and serological cross-reactivity studies. Techniques of recombinant protein expression and protein electrophoresis. Protein purification by HPLC.

2007: Industrial University of Santander, National Laboratory Chromatography and Agroindustrial Complex CENIVAM.

2004-2008: Training in extraction, characterization and evaluation of the antioxidant activity of essential oils and aromatic plants from Colombian. University of Cartagena, Cartagena de Indias, Colombia. Faculty of Pharmaceutical Chemistry. Environmental and Computational Chemistry Group. Training in techniques for assessing acute toxicity against *Artemia franciscana*, hemolytic activity, antioxidant activity and antimicrobial activity of plant extracts.

PUBLICATIONS

-**Cantillo JF**, Puerta L, Puchalska P, Lafosse-Marin S, Subiza JL, Fernández-Caldas E. Allergenome characterization of the mosquito *Aedes aegypti*. Allergy. 2017;00:1–11. https://doi.org/10.1111/all.13150

-**Cantillo, J.F.**, L. Puerta, S. Lafosse-Marin, J.L. Subiza, L. Caraballo, E. Fernández-Caldas. Allergens involved in the cross-reactivity of *Aedes aegypti* with other arthropods. Ann Allergy Asthma Immunol. 2017. Accepted for publication.

-Fernández-Caldas, E., Cases, B., El-Qutob, D., **Cantillo, J.F.** Mammalian raw materials used to produce allergen extracts. 2017. Ann Allergy Asthma Immunol. Accepted for publication.

-**Cantillo, J.F.**, L. Puerta, S. Lafosse-Marin, J.L. Subiza, L. Caraballo, E. Fernández-Caldas. Identification and characterization of IgE-binding tropomyosins in *Aedes aegypti*. 2015. Int Arch Allergy Immunol. 2016. 170(1):46-56.

-**J.F. Cantillo**, L. Puerta, S. Lafosse-Marin, L. Caraballo, E. Fernández-Caldas. Allergen characterization of *Aedes aegypti* by a proteomic approach. J. Allergy and Clin. Immunol. 2015. 135(2):AB105.

-**Cantillo, J.F.**, Puerta, L., Lafosse-Marin, S., Fernández-Caldas, E. Mosquito allergy in the tropics: involvement of tropomyosin and cross-reactivity with other arthropods. 2014. Allergy. 69 (Suppl. 99). 432.

-**J.F. Cantillo**, L. Puerta, E. Fernández-Caldas. S. Lafosse-Marin. Characterization of the allergenic activity of Tropomyosin from *Aedes aegypti*. J. Allergy and Clin. Immunol. 2014. 133(2):AB102

-**Cantillo, J.**, Fernández-Caldas, E. and Puerta, L. Immunological aspects of the immune response induced by mosquito allergens. Int Arch Allergy Immunol. 2014;165(4):271-82

-**Cantillo, J.F.**, Puerta, L. Nuevos esquemas inmunoterapia específicas con alergenos. 2010. Biomédica; 30:440-453

-**Cantillo, J.**, Güette, J., Baldiris R., Jaramillo B. y Olivero, J. Evaluación de la toxicidad aguda (CL50) de los extractos acuoso, diclorometano y metanólico parcial de Justicia secunda (Vahl.) frente Artemia franciscana. Scientia Et Técnica, abril, año/vol. XIII, número 033. pp. 257-258.

ABSTRACTS AND CONGRESS PARTICIPATION

-**Cantillo J.F.**, Puerta L., Lafosse-Marin S., Subiza J.L., Caraballo L., Fernandez-Caldas E. Molecular components involved in the IgE cross-reactivity between *Aedes aegypti* and arthropods. European Academy of Allergy and Clinical Immunology. Vienna, Austria. 2016

-**Cantillo J.F.**, Puerta L., Fernandez-Caldas E., Subiza J.L., Wöhrl S., Ebner C., Keller W., Resch Y., Vrtala S., Bohle B. Tropomyosin variants from the mosquito *Aedes aegypti* and the house dust mite *Dermatophagoides pteronyssinus* cross-react. European Academy of Allergy and Clinical Immunology. Vienna, Austria. 2016.

-**Cantillo, J.F.**, Puerta, L., Puchalska, P., Subiza, J.L., Lafosse-Marin, S., Fernández-Caldas, E. Identification of new IgE-binding proteins from the mosquito *Aedes aegypti*. European Academy of Allergy and Clinical Immunology. Barcelona, Spain. 2015

-**J.F. Cantillo**, L. Puerta, S. Lafosse-Marin, L. Caraballo, E. Fernández-Caldas. Allergen characterization of *Aedes aegypti* by a proteomic approach. American Academy of Allergy, Asthma and Immunology. Los Angeles, USA. 2015

-**Cantillo, J.F.**, Puerta, L., Lafosse-Marin, S., Fernández-Caldas, E. Mosquito allergy in the tropics: Involvement of tropomyosin and cross-reactivity with other arthropods. European Academy of Allergy and Clinical Immunology. Copenhague, Denmark. 2014

-**J.F. Cantillo**, L. Puerta, S. Lafosse-Marin, E. Fernandez-Caldas. Characterization of the allergenic activity of tropomyosin from *Aedes aegypti*. American Academy of Allergy, Asthma and Immunology. San Diego, USA. 2014.

-Dalgys Martinez, Marlon Munera, **Jose Cantillo**, Leonardo Puerta, Luis Caraballo. A Fusion Protein Designed with Segments of Different Allergens of *Blomia tropicalis*. J. Allergy and Clin. Immunol. 2012. 131(2). AB11.

-Puerta L, Martínez D, Múnera M, **Cantillo J**, Caraballo L. Recombinant protein assembling epitopes from different allergens of *Dermatophagoides pteronyssinus*. European Academy of Allergy and Clinical Immunology Congress. Geneva, Zwitzerrland. 2012

-**Cantillo J**, Puerta L, Caraballo L. Aplicación de proteínas de fusión en el diagnóstico de alergias por ácaros. CD: Scientific convention – University of Cartagena. Ciencia y tecnología a la altura de los tiempos. 2010.

-**Cantillo J**, Puerta L, Caraballo L. Obtención y purificación de dos alergenos recombinantes expresados en levadura. CD: Scientific convention – University of Cartagena. Ciencia y tecnología a la altura de los tiempos. 2010.

-**Cantillo J**, Puerta L, Caraballo L. Producción y purificación de dos alergenos recombinantes del ácaro *Blomia tropicalis* expresados en levadura. CD: VII Congreso de Ciencias Farmacéuticas XI Simposio Colombiano de Ciencia y Tecnología Cosmética. Cartagena de Indias, Colombia, 2010.

-**Cantillo J**, Puerta L, Caraballo L. Diseño, expresión y reactividad IgE de una proteína con epítopes de diferentes alergenos del ácaro *Dermatophagoides pteronyssinus*. Segundo encuentro de investigadores en inmunología. Universidad de los Andes. Mayo de 2010.

-**Cantillo J**, Güette J, Baldiris R, Jaramillo B, Olivero J. Evaluación de la toxicidad aguda (CL50) frente a *Artemia franciscana* y la actividad hemolítica de los extractos acuosos, en diclorometano y metanólico parcial de *Justicia secunda* (Vahl.). IX Congreso colombiano de fitoquímica. Mayo de 2007.

-**Cantillo J**, Güette J, Baldiris R, Jaramillo B, Olivero J. Evaluación de la concentración letal media frente a *Artemia franciscana*, actividad antioxidante total y actividad hemolítica de extractos acuosos y metanólico parcial de *Justicia secunda* (Vahl). VIII Congreso nacional de estudiantes de química farmacéutica. Octubre de 2007.

-**Cantillo J**, Güette J, Baldiris R, Olivero J. Evaluación de la actividad hemolítica y toxicidad aguda (CL50) frente a *Artemia franciscana* de extractos de *Justicia secunda* (Vahl). V Congreso de ciencias farmacéuticas. Octubre de 2006.

BOOK CHAPTERS

-**José Cantillo** and Leonardo Puerta. From Molecular Cloning to Vaccine Development for Allergic Diseases. An Integrated View of the Molecular Recognition and Toxinology - From Analytical Procedures to Biomedical Applications. 2013. Publisher: InTech. Page: 291-321.

-Sánchez J, Estarita J, **Cantillo J**, Sánchez A, Eggs Allergy: When and Why? Eggs: Nutrition, Consumption and Health 2011. Ed: NOVA publishers. Initial page: 99.

RESEARCH PROJECTS

-Obtaining fusion proteins: Application in diagnosis of allergies caused by mites. 2009. Co-researcher. Financing agency: Administrative department of science, technology and innovation (Colciencias).

-Recombinant allergens for diagnosis and immunotherapy: Production and purification of two recombinant allergens expressed in yeast. 2009. Co-researcher. Financing agency: University of Cartagena, Cartagena de Indias-Colombia.

-Characterization of *Blomia tropicalis* allergens from a cDNA library. 2007. Co-researcher. Financing agency: University of Cartagena, Cartagena de Indias-Colombia.

PATENTS

- “Fusion proteins with representation of different allergens: Vaccine proposal for mite allergens”. Puerta L., Caraballo L, Cantillo JF. European Patent: EP 2727934 B1. Granted Jun.15, 2016.

- “Fusion Proteins representing different allergens and vaccine against allergy to mites”. Puerta L, Caraballo L, Cantillo JF. Patent US: 9,163,069 B2, Oct. 20, 2015.

SKILLS

Spanish as a first language and fluent English (TOEFL level 86). A goal oriented/results driven researcher with strong analytical and problem solving skills.

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I certify that all information entered by me in this resume is true and can be demonstrated at any time