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Allergenome characterization of the mosquito *Aedes aegypti*

Short title: *Aedes aegypti* allergenome

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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 *A. 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 less than 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.

INTRODUCTION

Allergic respiratory diseases affect 10 to 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) .

A. 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 non-salivary 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). *A. aegypti* has additional IgE-binding proteins that remain to be characterized. This insect contains at least 8 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 is 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.

MATERIALS AND METHODS

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 7 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 non-allergic 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 tuberculose (CDMMRT)”.

Total protein extraction

Defatted and lyophilized *A. aegypti* mosquito bodies material was purchased from GREER labs (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, 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 re-suspended with ReadyPrep 2-D rehydration/sample buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, 0.001% Bromophenol Blue). Protein concentration was measured using RC DC Protein Assay (Bio-Rad).

2-D Electrophoresis

Isoelectric focusing (IEF) was performed using 11 cm, pH 3–10 linear IPG strips (Bio-Rad). Strips were rehydrated with 200 μ L of rehydration buffer containing 200 μ g of protein, at room temperature. Proteins were focused at 50 μ A/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 M urea, 20% v/v glycerol, 2% w/v SDS, 0.375 M Tris-HCl (pH 8.8) and 2% DTT, followed by equilibration buffer 2 containing 6 M urea, 20% v/v glycerol, 2% w/v SDS, 0.375 M Tris-HCl (pH 8.8) and 2.5% w/v iodoacetamide. Afterward, IPG strips were washed with SDS-PAGE running buffer (25 mM Tris, 192 mM 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.

SDS-PAGE, IgE immunoblot and inhibition analysis

Proteins from *A. aegypti* were separated by 1-dimensional electrophoresis. Proteins from the 1-dimensional and 2-dimensional gel electrophoresis were electro-transferred 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 1-dimensional gel electrophoresis were electro-transferred 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.

Selection of specific IgE binding spots

Coomassie stained and immunoblotted 2-D gels were compared using GeneTools image analysis software. 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 was 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.

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.

Tryptic digestion

Gel spots were washed with 50 mM ammonium bicarbonate, incubated with DTT (10 mM in ammonium bicarbonate) at 56 °C for 20 min, followed by iodoacetamide (50 mM in ammonium bicarbonate) for 20 min in the dark. Spots were dried and incubated with trypsin (12.5 µg/mL, in ammonium bicarbonate, 10 µL) for 20 min 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).

PMF analysis

PMF 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). One µL 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 Biotoools 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* (16654 sequences; 7684696 residues). For identifications where one peptide was assigned to protein, data was researched against Swissprot (Uniprot) (546439 sequences; 194445396 residues), and NCBI (51471198 sequences; 18460256539 residues) databases in order 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 2 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).

PFF analysis

Separation of tryptic peptides was performed using NanoAcquity nano-LC (Waters) equipment, on a Waters BEH C18 nano-column (200 mm x 75 µm ID, 1.8 µm). Peptide elution was performed using a chromatographic ramp from 5 to 60% of mobile phase B during 30 min, and flow rate of 300 nL/min. Mobile phase A was water containing 0.1% v/v formic acid, while mobile phase B was ACN containing 0.1% v/v formic acid. MS data was 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 post-acquisition 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, London, UK) against a custom database filled with all the UNIPROT entries corresponding to *A. aegypti* (16654 sequences; 7684696 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.

Allergenicity test and bioinformatic analysis

Frequency of IgE-binding for each spot was determined in the immunoblots with all individual sera. Bioinformatics analysis to predicts potential allergenicity were performed using: 1) 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, 2) 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 3) Algpred (<http://www.imtech.res.in/raghava/algpred/>) (15) which predicts based on similarity of known epitope with any region of protein.

RESULTS

Proteomic identification of IgE-reactive proteins

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

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 SwissProt, 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. Spots no. 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 (no. 21 to 23) and Voltage-dependent anion-selective channel activity (no. 24). Variants or isoforms of “Arginine or creatine kinase” (Uniprot code: Q1HR67), (no. 6 to 12) and Glyceraldehyde-3-phosphate dehydrogenase (GPDH) (Uniprot code: J9HYM2) (no. 13 to 17). A hypothetical protein (Uniprot code: Q16TN9) was also identified (no. 25).

Relevance of tropomyosin family in the IgE-binding protein repertoire of A. 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 1 D).

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 6 proteins reacted with <33.3% of

the sera. “Arginine, or creatine kinase” and 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 colour 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).

Novel A. aegypti allergens

The WHO/IUIS Allergen Nomenclature Sub-committee (www.allergen.org) has assigned official names to 5 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 *D. 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).

Discussion

The proteome and transcriptome of *A. aegypti* have been studied to: a) discover potential receptors for insecticidal proteins (21, 22), b) understand the mechanisms of infection for some viruses (23, 24), c) study the molecular mechanisms associated with mosquito bites (25, 26) and d) 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 *D. 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 and not only in the saliva, since 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 4 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 since no unique peptides were obtained. However, we hypothesize that spot no. 3 corresponds to Aed a 10.0201 (Q17H80). Proteins on spots no. 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 data base; 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 merguensis* (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 contains the complete repertoire of IgE epitopes from the mosquito tropomyosin family (11). The results from our study confirmed this observation, since rAed a 10.0101 produced complete inhibition of the IgE-binding to the 32kDa 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 to the cutaneous reactions that appear after mosquito bites (10, 42-45). In this study, we did not detect these allergens since 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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CONFLICT OF INTEREST STATEMENTS

JFC Reports no conflict of interests in relationship with this paper.

LP Reports no conflict of interests in relationship with this paper.

PP Reports no conflict of interests in relationship with this paper.

SLM Reports no conflict of interests in relationship with this paper.

JLS Reports no conflict of interests in relationship with this paper.

EFC Reports no conflict of interests in relationship with this paper.

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TABLES

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 <i>A. aegypti</i> (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 SPT with extracts of *A. communis*, *D. pteronyssinus*, *D. farinae*, *B. tropicalis*, Shrimp and Cockroach.

Table 2. Protein identification of specific IgE-binding proteins from *A. 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 ²
1	Q03168 ¹	Lysosomal aspartic protease	N.A.	May degrade organelles	310	76	26	---	32.6/5.2	41.8/5.2
2	Q17H75 ¹	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 ¹	AAEL011197-PA	N.A.	ATP-binding ³	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 ³	120	34	16	9/37	69.0/5.6	72.3/5.1
6	Q1HR67	Arginine or creatine kinase	N.A.	Kinase ATP-binding ³	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 ³	123 ⁴	38	4	1/6	32.5/7.5	35.4/8.3
14					115 ⁴	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 ⁴	33	4	1/8	32.3/9.7	
18	Q16XK7 ¹	AAEL008844-PA	N.A.	Calcium ion binding ³	938	58	72	---	17.9/4.9	21.9/4.6
19	Q17H75 or Q17H76 ¹	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 activity ³	99 ⁵	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	86	34	12	2/3	24.7/7.7	30.7/8.63

				activity ³						
25	Q16TN9	AAEL010180-PA	N.A.	Unknown	98	33	12	3/28	27.4/5.4	23.5/4.7

¹Protein identified using nLC-MS/MS

²Theoretical MW and pI was assigned using Compute MW/pI tool available on ExPasy

³Information from Gene Ontology

⁴Identity confirmed by searching data against SwissProt database

⁵Identity confirmed by searching data against NCBI database

N.A. Not previous allergen name assigned in IUIS

Table 3. Allergenicity of *A. aegypti* components based on the serum IgE reactivity in 15 individuals with respiratory allergies.

Spot	Protein name	Patient															Frequency of reactivity (%)	Predicted as allergen by
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1	Lysosomal aspartic protease	+		+		+		+				+			+		40	Non evaluated
2	Tropomyosin	+		+	+	+		+			+	+	+		+		60	Allertop, PREAL, ALGPRED
3	Tropomyosin	+		+		+		+	+		+	+	+		+		60	Allertop, PREAL, ALGPRED
4	AAEL011197-PA																0	Non evaluated
5	HSC-70	+		+		+		+		+	+	+	+		+		60	Allertop, PREAL, ALGPRED
6-12	Arginine or creatine kinase							+							+		13.3	Non evaluated
13-17	GPDH						+										6.7	Non evaluated
18	AAEL008844-PA		+	+		+		+									33.33	PREAL, ALGPRED
19-20	Tropomyosin			+				+			+	+	+		+		40	Non evaluated
21-23	AAEL006070-PA																40	Non evaluated
24	AAEL001872-PA								+	+				+		+	33.3	Allertop, ALGPRED
25	AAEL010180-PA							+			+	+	+				33.3	Non evaluated

Table 4. Summary of peptides from the five new allergens of *A. aegypti*, identified by mass spectrometry analysis.

Spot	Protein name	Allergen name	Identified peptides	Peptides specific for <i>A. aegypti</i>	Peptides specific for <i>A. aegypti</i> 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 VTHAVVTPAYFNDAQR DNHLLGKFDLTGIPPAPR KVTHAVVTPAYFNDAQR IEIESFYEGDDFSETLTR		IEIESFYEGDDFSETLTR
18	AAEL008844-PA	Aed a 5	VDDSYNQLVSDENK AFIDAHYQMMDINNDGLVSIEEYR YQELYAQFMGNESDK KVDDSYNQLVSDENK ALWDEISALADLDHDGK GGITLQR VDDSYNQLVSDENKR SYSDFPK IAMDDIK LNEYR	VDDSYNQLVSDENK KVDDSYNQLVSDENK ALWDEISALADLDHDGK VDDSYNQLVSDENKR	AFIDAHYQMMDINNDGLVSIEEYR YQELYAQFMGNESDK
24	AAEL001872-PA	Aed a 6	EFGGLIYQR VTANNFALGYSTGDFVLHTNVNDGR	VTANNFALGYSTGDFVLHTNVNDGR	
25	AAEL010180-PA	Aed a 7	FIQNELPR ETLVTLLLR FVLLHEFIR	ETLVTLLLR FVLLHEFIR	

Abbreviations:

2DE: Two-dimensional gel electrophoresis

ACN: Acetonitrile

BSA: Bovine serum albumin

CDMMRT: Comite departemental de la Martinique contre les maladies respiratoires et la tuberculose

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

IEF: Isoelectric focusing

IEP: Isoelectric point

kDa: Kilo Dalton

MALDI: Matrix Assisted Laser Desorption Time of Flight

MS: Mass spectrometry

nLC: nano-liquid chromatography

PBS: Phosphate-buffered saline

PFF: Peptide fragment fingerprint

PMF: Peptide mass fingerprint

SDS: Sodium dodecyl sulfate

SPT: Skin prick test

ToF: Time of flight

WHO/IUIS: World Health Organization and International Union of Immunological Societies

Figure legends:

Figure 1. Two dimensional gel electrophoresis of total proteins from *A. aegypti*. (A) Spots were identified by Coomassie stain, and (B) specific IgE immunoblot using pooled sera. (C) Serum from a non-allergic 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.

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

