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Permalink
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Journal
Allergy, 71(5)

ISSN
0001-5148

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et al.

Publication Date
2016-05-01

DOI
10.1111/all.12812

Peer reviewed

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Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI

Allergy

ORIGINAL ARTICLE EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Mosquito salivary allergen Aed a 3: cloning, comprehensive molecular analysis, and clinical evaluation

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To cite this article: Peng Z, Xu WW, Sham Y, Lam H, Sun D, Cheng L, Rasic NF, Guan Q, James AA, Simons FER. Mosquito salivary allergen Aed a 3: cloning, comprehensive molecular analysis, and clinical evaluation. *Allergy* 2016; **71**: 621–628

Keywords

Aed a 3; *Aedes aegypti*; insect allergy; mosquito; salivary allergen/antigen.

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Accepted for publication 19 November 2015

DOI:10.1111/all.12812

Edited by: Reto Crameri

Abstract

Background: Allergic reactions to mosquito bites are an increasing clinical concern. Due to the lack of availability of mosquito salivary allergens, they are underdiagnosed. Here, we reported a newly cloned mosquito *Aedes* (*Ae.*) *aegypti* salivary allergen.
Methods: A cDNA encoding a 30-kDa *Ae. aegypti* salivary protein, designated Aed a 3, was isolated from an expression library. The full-length cDNA was cloned into a baculovirus expression vector, and recombinant Aed a 3 (rAed a 3) was expressed, purified, and characterized. Skin prick tests with purified rAed a 3 and *Ae. aegypti* bite tests were performed in 43 volunteers. Serum rAed a 3-specific IgE levels were measured in 28 volunteers.
Results: The primary nucleotide sequence, deduced amino acid sequence, and IgE-binding sites of Aed a 3 were identified. rAed a 3-selected antibodies recognized a 30-kDa *Ae. aegypti* saliva protein. rAed a 3 bound IgE in mosquito-allergic volunteers and the binding could be inhibited by the addition of natural mosquito extract dose dependently. Immediate skin test reactions to rAed a 3 correlated significantly with mosquito bite-induced reactions. Of the bite test-positive volunteers, 32% had a positive rAed a 3 skin test and 46% had specific IgE. No bite test-negative volunteers reacted to rAed a 3 in either the skin tests or the IgE assays, confirming the specificity of the assay.
Conclusions: Aed a 3 that corresponds to the Aegyptin protein is a major mosquito salivary allergen. Its recombinant form has biological activity and is suitable for use in skin tests and specific IgE assays in mosquito-allergic individuals.

Mosquitos are a global problem, not only because they facilitate the transmission of potentially fatal diseases such as dengue and yellow fever, but also because their bites can cause large local skin reactions and, less commonly, systemic reactions including urticaria, angioedema, and anaphylaxis (1–8).

Abbreviations

Ae., *Aedes*; apap, antiplatelet aggregation protein; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; PCR, polymerase chain reaction; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; Sf9, *Spodoptera frugiperda* f9 cells.

Additionally, inhalation of mosquito allergens can cause asthma and/or allergic rhinoconjunctivitis symptoms in sensitized individuals (9, 10). In patients with Epstein–Barr virus-associated T-/natural-killer cell-associated lymphoproliferative disorders, reactions to mosquito bites involve an intense skin reaction associated with systemic symptoms (11). Mosquito saliva proteins elicit both IgE-mediated immediate hypersensitivity and lymphocyte-mediated delayed hypersensitivity (3, 10, 12). IgG, especially IgG4, also may be involved in the development of mosquito bite-induced skin reactions (1, 3).

Currently, only mosquito whole-body extracts, containing multiple antigens not present in mosquito saliva, but few

actual salivary allergens (13), are commercially available for diagnosis and immunotherapy in mosquito allergy. We compared the UniCAP assay that utilizes mosquito whole-body extract with our mosquito saliva-capture enzyme-linked immunosorbent assay (ELISA) for the measurement of mosquito-specific IgE. The results indicated that the mosquito saliva-capture ELISA is significantly more sensitive and specific than the UniCAP test (14). However, collection of mosquito saliva is time-consuming, labor intensive, and impractical. Utilization of molecular techniques to clone and express pure allergens and even allergen-derived peptides has been successful for treating allergic patients (15–17).

Aedes (Ae.) aegypti is an important mosquito species with a worldwide cosmotropical distribution. We have previously expressed, purified, and clinically evaluated two recombinant *Ae. aegypti* mosquito salivary proteins (18, 19) from two previously isolated cDNAs (20, 21). These two recombinant proteins bind to human IgE and elicit skin reactions in people who are allergic to mosquito bites. In accordance with allergen nomenclature, the 68-kDa protein (biochemical name 'apyrase') is called Aed a 1 (18) and the 37-kDa protein (biochemical name 'D7') is called Aed a 2 (19).

Here, we report the isolation of a new cDNA encoding a 30-kDa IgE-binding protein from an *Ae. aegypti* salivary gland cDNA expression library using mouse anti-*Ae. aegypti* saliva serum and the evaluation of its clinical relevance in volunteers with *Ae. aegypti* bite tests. This 30-kDa salivary protein is designated as Aed a 3.

Methods

Participants

This project was approved by the University of Manitoba Research Ethics Board. The participants gave written, informed consent before study enrollment. Forty-three healthy volunteers (22 women and 21 men), aged 20–54 with a history of skin reactions to mosquito bites that ranged from no reaction to large local reactions, were recruited from people who responded to advertisements. No participants had taken antihistamines within 5 days prior to the study. All 43 volunteers had a bite test from a laboratory-reared mosquito and a skin prick test with rAed a 3; 23 volunteers also had a 10-ml blood sample taken for the assay of rAed a 3-specific IgE.

Mosquitos, mosquito antigens, and antisaliva sera

The *Ae. aegypti* colony was obtained from the Department of Entomology, University of Manitoba, and maintained in our laboratory. Mosquito saliva extracts and head and thorax extracts were prepared as described previously (22, 23). Mouse anti-*Ae. aegypti* saliva serum was produced in our laboratory by immunization of mice with *Ae. aegypti* saliva and adjuvants (24). Mosquito-allergic human serum was obtained by pooling sera from participants who had large local reactions to mosquito bites and high titers of mosquito salivary gland-specific IgE. Control human serum was obtained by pooling sera from participants with negative mosquito bite tests.

Molecular cloning, DNA sequencing, and molecular modeling

The salivary gland λgt11 cDNA library of adult female mosquito *Ae. aegypti* (provided by Dr. Anthony James, University of California, Irvine, CA, USA) was screened with mouse anti-*Ae.* aegypti saliva serum, and two sets of PCR primer pairs were designed to combine two overlapping cDNA clones into a full-length Aed a 3 cDNA clone (Data S1). The cDNA was subcloned into the vector pBluescript II SK (Stratagene, La Jolla, CA, USA) and sequenced using a kit supplied by US Biochemicals (Cleveland, OH, USA).

The Amersham Staden Plus software package was used to analyze the nucleotide sequence of the cDNA for open reading frames (ORF) and deduce the amino acid sequence. To identify the antigenic epitope, homology modeling of the C-terminal sequence of Aed a 3 was carried out using the Schrodinger modeling package (Schrodinger, LLC, New York, NY, USA). The X-ray crystallographic structure of Anopheles antiplatelet aggregation protein (apap) fragment in complex with a mouse Fab antibody (PDB 4OKV)(25) was used as the structural template for homology modeling of the C-terminal sequence of Aed a 3 187-253. All missing side chains and hydrogen atoms were added using the standard protein preparation protocols at physiological pH, followed by energy minimization in implicit solvent to optimize all hydrogen-bonding networks.

Expression and purification of rAed a 3

The full-length protein encoded by the Aed a 3 cDNA was expressed using a baculovirus expression system (Data S1). Concentrated rAed a 3 supernatant was loaded onto a DEAE–Sephacel column and then eluted with a linear NaCl gradient (0–0.6 M in Tris buffer). Protein concentrations were monitored by OD_{595} nm and rAed a 3 content assayed using ELISA and immunoblots (Data S1). Fractions containing rAed a 3 were pooled and concentrated to a final protein concentration of 0.2 mg/ml for use in skin tests and serum rAed a 3-specific IgE assay.

Preparation of fusion protein-selected antibodies

Nitrocellulose membranes absorbed previously with the fusion protein produced by the cloned gene were used to purify specific antibodies from mouse anti-mosquito saliva serum and also from mosquito-allergic human serum using the methods reported previously (Data S1). These clone-specific antibodies were used in immunoblot analyses to detect their binding to the native protein present in *Ae. aegypti* saliva.

Immunoblotting

To identify which protein in the mosquito saliva reacts with the fusion protein-selected mouse antibodies, proteins in *Ae. aegypti* saliva extract were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting analyses were performed as described previously (22). Mem-

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branes were incubated with fusion protein-selected mouse antibodies or mouse antisaliva serum as a positive control or PBST containing 1% BSA, followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Calbiochem Corporation, San Diego, CA, USA) and then with ECL detecting reagents (Amersham Life Science, Buckinghamshire, UK). For the human IgE-binding test, the membranes containing saliva proteins were incubated with fusion protein-selected human antibodies or a mosquito-allergic serum or the control buffer, followed by incubations with monoclonal anti-human IgE (ascites, clone No. 7.12, provided by Dr. A. Saxon, University of California, Los Angeles, CA, USA), then with peroxidase-conjugated goat anti-mouse IgG and finally with ECL detecting reagents.

To identify whether rAed a 3 reacts with saliva-induced antibodies in humans, proteins in purified rAed a 3 and in *Ae. aegypti* saliva (positive control) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with human mosquito-allergic serum, followed by incubation with monoclonal anti-human IgE and then with alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Lab Inc., West Grave, PA, USA). After washing, the color was developed by incubation of the membranes with an alkaline phosphatase substrate solution.

ELISA and ELISA inhibition tests

An ELISA was developed to detect rAed a 3-specific IgE in human sera using the technique described previously (19, 26). In brief, microtiter plates were coated with 0.05 μg/well of purified rAed a 3 protein. After blocking the free binding sites, the plates were incubated with human sera (diluted 1 : 20). The bound serum Aed a 3-specific IgE was detected by incubation of the plates with goat anti-human IgE (a gift from Dr. N.F. Adkinson Jr., The Johns Hopkins Allergy and Asthma Centre, Baltimore, USA), followed by incubations with alkaline phosphatase-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Lab. Inc.) and finally with an alkaline phosphatase substrate solution. Optical absorbance at 410 nm was read using a THERMO-max microplate reader.

An ELISA inhibition test was performed to determine whether the binding of human IgE to rAed a 3 could be inhibited by natural Aed a 3 in the saliva and to determine the specificity of the rAed a 3-specific IgE assay. The mosquito head and thorax extract (1.48 mg/ml) was diluted 10-fold. Each dilution was mixed with an equal volume of a 1 : 25 diluted mosquito-allergic serum. Dilution mixed with PBST served as a control. After incubation at 4°C overnight, rAed a 3-specific IgE contained in the mixtures was measured by ELISA as described above.

Mosquito bite tests and skin prick tests

In 43 healthy volunteers, the mosquito bite test was performed on the volar aspect of the left forearm using a laboratory-raised *Ae. aegypti* female mosquito (27). Skin prick tests were carried out on the right forearm with purified rAed a 3 (0.2 mg/ml), histamine (1 mg/ml) as a positive control, and saline as a negative control. The histamine-induced wheal and flare were traced with pen after 10 min and the other test sites were traced after 20 min and at 24 h post-test. All tracings were transferred to paper and the areas measured (27). An immediate wheal or a delayed induration of $\geq$0.3 cm^2 was considered to be a positive reaction.

Statistical analyses

Values were expressed as mean $\pm$ SD. The unpaired Student *t*-test was used to compare the differences. Linear regression analyses were used to determine the correlations. All statistical analyses were performed using GraphPad Prism version 5 (www.graphpad.com/scientific-software/prism/). *P* values $<$0.05 were considered statistically significant.

Results

cDNA cloning and sequence analysis

A total of 39 positive plaques were identified following screening of ~120 000 plaques from the whole-salivary gland cDNA library of adult *Ae. aegypti* with mouse antisaliva serum (Data S1). Three clones with different sizes of cDNA inserts were subcloned. One clone (λAA22) with a complete 3′ terminus with a poly-A tail was selected for further analysis. This fragment was determined to contain the major coding region of the 30-kDa protein because the cloned cDNA (0.73 kb) has an open reading frame of 217 amino acids, the molecular weight of which is estimated to be 26 kDa. This clone lacked the 5′ terminal sequence because no initiation codon was observed.

Gene amplification techniques were used to clone the 5′ terminal fragment of the full-length cDNA. The full-length cDNA is 0.85 kb, encodes a protein of 254 amino acid residues with a relative molecular weight of ~30 kDa. In accordance with allergen nomenclature, this 30-kDa protein was named as Aed a 3 that corresponds to the Aegyptin protein (41). Both Aed a 3 and Aegyptin proteins are encoded by the same gene. The primary nucleotide sequence of the Aed a 3 cDNA and its deduced amino acid sequence are described in Data S1, and the sequence data were deposited in GenBank in 1997 (AF001927, http://www.ncbi.nlm.nih.gov/nuccore/AF001927).

Molecular antigen-binding site analysis

The X-ray-resolved crystallographic structure of a mosquito (anopheline) antiplatelet aggregation protein (apap) fragment was identified using sequence analysis (25). This apap fragment has 45% sequence similarity to the C-terminal region of Aed a 3 (amino acids 187-253) (Fig. 1A). The structure of the apap fragment is resolved in complex with the mouse Fab antibody at 1.8Å resolution and consists primarily of two alpha helices connected by a turn region with two disulfide bridges located at the two opposing ends of each helix.

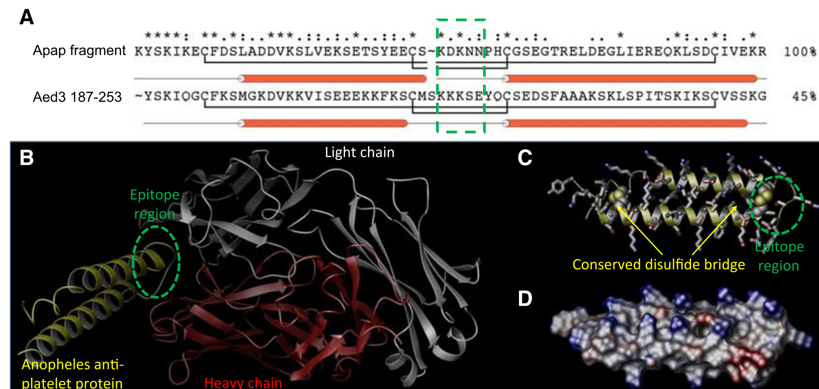

Figure 1 Homology model of the Aed a 3 C-terminal region. (A) Sequence alignment between the apap fragment and Aed a 3 C-terminal region. The two conserved disulfide bridges are highlighted with the antigenic epitope region boxed in green. (B) X-ray structure of apap fragment (yellow) in complex with mouse FAB antibody consisting of a light chain (white) and a heavy chain (red). (C) Homology model of the Aed a 3 C-terminal region 187-253 with structurally conserved disulfide bridges. The antigenic epitope region is highlighted in green. (D) Electrostatic potential surface model of the Aed a 3 C-terminal region with the positively and negatively charged surfaces in blue and red, respectively.

Examination of the antigen-binding site with the nearest apap residues identifies the KDKNN sequence motif located at the interhelical turn as the antigenic epitope site for antigen binding (Fig. 1B). Based on these observations, homology modeling of the C-terminal region of Aed a 3 was carried out. The final model showed that all four cysteine residues essential for the formation of the two disulfide bridges in apap are conserved within the Aed a 3 C-terminal region (Fig. 1C). There is >30% reduction in the number of ionizable residues located on the surface of Aed a 3 C-terminal region as compared to apap, two-thirds of which comprise primarily lysine residues (Fig. 1D). Subsequently, the KKKSE sequence motif located within the turn region was identified as the most probable antigenic epitope region for antigen binding.

IgE binding property of the 30-kDa protein

Both the mouse IgG (A) and human IgE (B) antibodies selected by the λAA22 clone fusion protein recognize a 30-kDa protein in the saliva (Fig. 2, lane 2). This indicates that the cDNA we isolated encodes the 30-kDa salivary protein and that it can induce a specific IgG response in mice and a specific IgE response in mosquito-allergic volunteers.

As shown in Fig. 3, the Aed a 3 fusion protein binds directly to IgE in mosquito-allergic human sera. Further, the binding of serum rAed a 3-specific IgE was successfully inhibited in the ELISA inhibition test in a dose-dependent manner by the addition of mosquito head and thorax extract (Fig. 4). This supports the conclusions that the recombinant Aed a 3 allergen and native Aed a 3 allergen in the salivary gland extract have identical antigenicity and that the rAed a 3-captured ELISA is specific for the detection of Aed a 3-specific IgE in human serum.

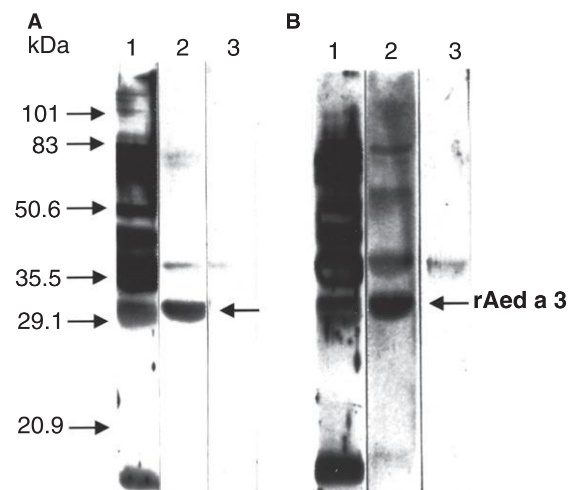

Figure 2 Fusion protein-selected antibodies recognize a 30-kDa *Ae. aegypti* salivary protein. A. Mouse antibodies. *Ae. aegypti* saliva proteins were separated by SDS-PAGE and immunoblotted with mouse antisaliva serum (lane 1), fusion protein-selected mouse antibodies (lane 2), or PBST (lane 3), followed by incubation with peroxidase-conjugated goat anti-mouse IgG. B. Human antibodies. *Ae. aegypti* salivary proteins were separated by SDS-PAGE and immunoblotted with a pooled human mosquito-allergic serum (lane 1), fusion protein-selected human antibodies (lane 2), or PBST (lane 3), followed by incubation with mAb anti-human IgE.

Skin testing and clinical relevance

No systemic reactions were occurred after the mosquito bite tests or skin tests. Twenty-eight of the 43 participants (65%) had a positive immediate skin reaction in the bite test, while

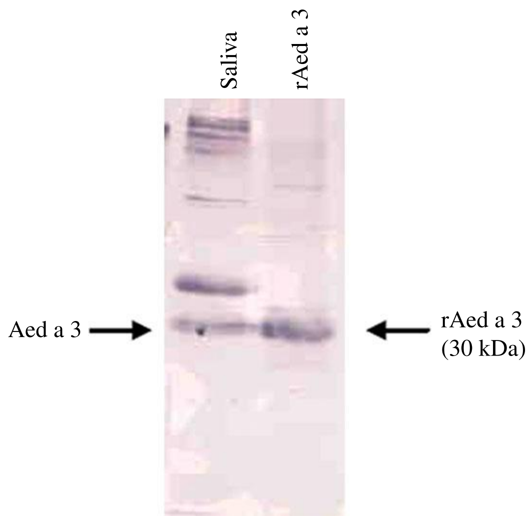

Figure 3 rAed a 3 protein binds to the IgE in mosquito-allergic human serum. Proteins in *Ae. aegypti* saliva (lane 1) and purified rAed a 3 (lane 2) separated by 10% SDS-PAGE were transferred to nitrocellulose membrane. The membranes was immunoblotted with a pooled mosquito-allergic human serum and incubated with monoclonal anti-human IgE.

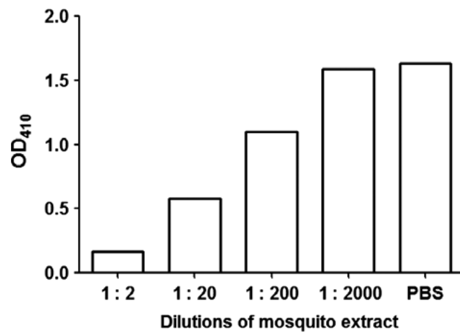

Figure 4 Binding of rAed a 3 to human IgE can be inhibited in a dose-dependent manner by a natural *Ae. aegypti* head and thorax extract containing salivary glands.

15 (35%) had a negative bite test. Of the 28 bite test-positive volunteers, 23 also had a positive delayed reaction. Similar to the bite reactions, skin prick tests with rAed a 3 also induced immediate wheals and flares and delayed indurations. Further, there were significant correlations between mosquito bite-induced and rAed a 3-induced skin immediate reactions ($r = 0.51$ for flares and $r = 0.43$ for wheals, P's <0.004) (Fig. 5).

rAed a 3-induced skin reactions were found only in the mosquito bite test-positive group and not in the bite test-negative group. For immediate reactions, two different positive cutoff levels were compared: the mean of bite test-negative individuals plus 1 standard deviation (SD) or 2SD. As shown in Fig. 6, the positivity rate of rAed a 3 is significantly higher in the bite test-positive group than in the bite

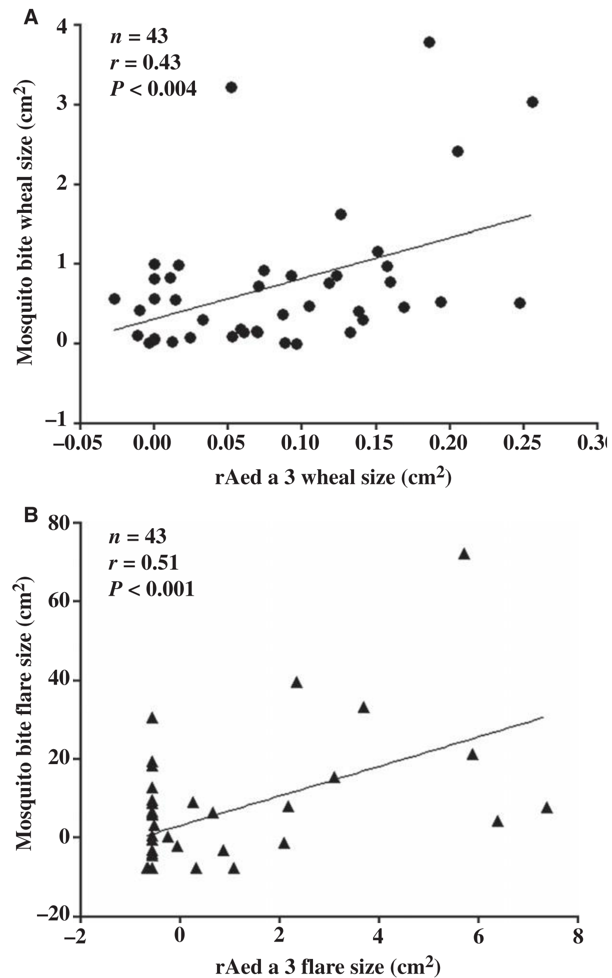

Figure 5 rAed a 3-induced skin flare and wheal sizes correlate significantly with those induced by mosquito bite tests.

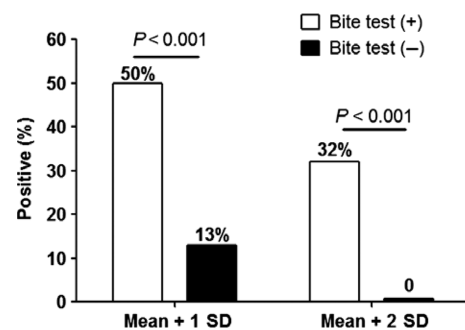

Figure 6 The positivity rate of skin prick testing with rAed a 3 is significantly higher in the *Ae. aegypti* bite test-positive group (n = 28) than in the *Ae. aegypti* bite test-negative group (n = 15). Two positive cutoff levels: the mean of rAed 3-induced skin positive reactions in the bite test-negative group plus 1SD or 2SD.

test-negative group for both cutoff levels: 50% *vs* 13% in 1SD cutoff and 32% *vs* 0% in 2SD cutoff. Using the method reported (28), diagnostic values of rAed a 3-induced immedi-

ate reactions were calculated as '137' and '132', respectively. Both cutoff levels had similar diagnostic values; the former is more sensitive and the latter is more specific (Table 1).

Due to the small amount of rAed a 3 introduced to the skin by prick testing, delayed reactions were only found in 3 individuals in the bite test-positive group.

Serum rAed a 3-specific IgE levels

Serum rAed a 3-specific IgE levels were significantly higher in the mosquito bite test-positive group than in the bite test-negative group (P = 0.0014) (Fig. 7). Using the geometric mean of the controls plus 2 SD as a cutoff level, ~46% (6/13) of the mosquito bite test-positive individuals exhibited a positive serum rAed a 3-specific IgE, while none of the bite test-negative individuals had a positive rAed a 3-IgE. This supports the conclusion that the assay is highly specific. The diagnostic value of the IgE assay is '146', which is higher than that of the skin tests ('137' or '132') (Table 1).

Table 1 Diagnostic values* of skin testing with rAed a 3 at different cut-off levels (%)

	Sensitivity	Specificity	Diagnostic value*
Mean + 1SD	50	87	137
Mean + 2SD	32	100	132

*The reliability of a diagnostic test, that is, its diagnostic value, is the Sum of its sensitivity and its specificity. It ranges from 'failure' at '0' to 'perfect' at 200%.

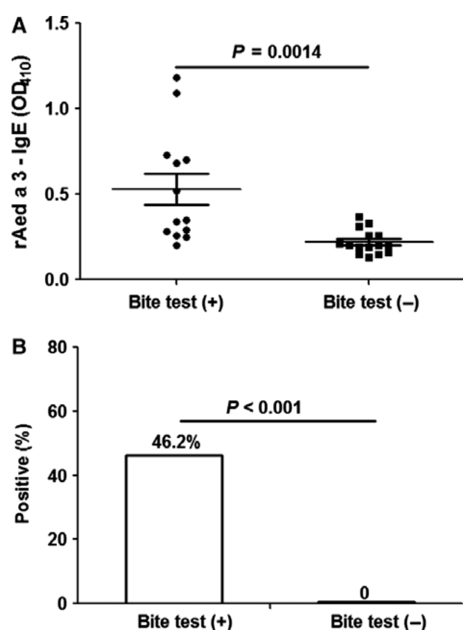

Figure 7 Serum rAed a 3-specific IgE levels were elevated significantly in the *Ae. aegypti* bite test-positive group (n = 13) compared with the bite test-negative group (n = 15). The geometric mean of the negative controls plus 2 SD was used as a cutoff level.

Discussion

The saliva of an adult female *Ae. aegypti* was reported in 1987 to contain a complex of as many as 20 proteins seen by sodium dodecylsulfate–polyacrylamide gel electrophoresis (29). In 2007, it was reported to contain 55 proteins coded by salivary gland-specific transcripts (30). Of them, at least eight proteins have been identified as allergens (22). In this study, we isolated the cDNA clone that encodes Aed a 3, a 30-kDa salivary allergen of *Ae. aegypti*.

Using molecular modeling, we identified an antigenic epitope region at the Aed a 3 C-terminal region. This IgE binding of the 30-kDa allergen (Aed a 3) was confirmed by several experiments. First, the fusion protein-selected mouse IgG and human IgE antibodies bound a 30-kDa protein present in *Ae. aegypti* saliva. Second, recombinant Aed a 3 directly bound to the IgE in mosquito-allergic serum that was induced by natural Aed a 3 in mosquito saliva in both immunoblotting and ELISA. More importantly, the binding of rAed a 3 to the IgE of a mosquito-allergic serum could be inhibited in a dose-dependent manner by the addition of natural Aed a 3 present in mosquito head and thorax extract. Further, rAed a 3 was able to induce both skin immediate and delayed reactions in the bite test-positive volunteers and the sizes of skin test reactions to rAed a 3 significantly correlated with the sizes of skin reactions to *Ae. aegypti* bite. As we anticipated, because rAed a 3 was expressed using a baculovirus/insect cell system that performs many of the post-translational modifications found in insect cells (31), it is not surprising that the rAed a 3 we produced had biological activity.

Recombinant allergens can not only serve as accurate diagnostic agents (32, 33), but also can facilitate safe and effective immunotherapy (15, 16). In this study, recombinant Aed a 3 proved to be an excellent candidate for use in *in vivo* and *in vitro* tests for the diagnosis of mosquito allergy, with 100% specificity in mosquito bite test-negative individuals. We have previously expressed and clinically evaluated two recombinant *Ae. aegypti* salivary allergens, rAed a 1 (18) and rAed a 2 (19) in addition to rAed a 3 in the study reported here. Preparation of additional recombinant mosquito salivary allergens is in progress. Development of assays using a combination of recombinant mosquito salivary proteins will eventually make it possible to diagnose mosquito allergy with accuracy and, if indicated, to provide immunotherapy.

In contrast to natural allergen extracts, recombinant allergens for immunotherapy can be produced in unlimited amounts with precise quality controls and modified to have more favorable characteristics including reduced IgE reactivity or enhanced immunogenicity (15–17). Currently, only mosquito whole-body extracts are available for use in skin tests, serum specific IgE tests, and immunotherapy which is effective in treating people who are sensitized to mosquito saliva (34–36). As the binding sites of rAed a 3 have been identified at the C-terminal region (amino acids 187-253), it might be easier to modify this allergen for new immunotherapy approaches.

In our previous studies, in which we used a cutoff level of the mean of controls plus 2SD, rAed a 1 induced a positive skin reaction in 29%(18) or 43% (37, 38) of the mosquito bite test-positive group and 0 in the bite test-negative group, while rAed a 2 induced a positive reaction in 11% of the bite test-positive group and 0 in the controls (37, 38). In another study of rAed a 2 (19), using mean plus 1SD of controls as a cutoff level, although rAed a 2 induced 43% positivity in the bite test-positive group, it also caused 5% false positivity in control group. These variations occur because of differences in the participants, allergen batches, and cutoff levels selected. In the present study, even at a cutoff level of mean plus 2SD, rAed a 3 induced 32% positivity rate for skin tests and 46% positivity for serum specific IgE assay in the bite test-positive group, and no positivity in the control group, confirming that rAed a 3 is a major allergen in *Ae. aegypti* saliva and that both skin prick and serum IgE tests using rAed a 3 are sensitive and specific in the assessment of mosquito allergy.

In addition to eliciting IgE responses, Aed a 3 plays an important role in mosquito feeding. After our isolation of the Aed a 3 cDNA (39), studies showed that the Aegyptin protein (the biochemical name of Aed a 3 also designated 30K b), secreted from the female *Ae. aegypti* salivary glands, plays a unique and critical role in the feeding success of this mosquito on vertebrate hosts (40, 41). This protein also binds collagen and inhibits platelet aggregation and adhesion (41). Future studies may identify additional roles for Aed a 3.

In conclusion, we have isolated and sequenced the cDNA coding for Aed a 3, a 30-kDa IgE-binding protein of mosquito *Ae. aegypti* saliva. Aed a 3 is a major allergen in *Ae. aegypti* saliva, eliciting positive reactions in 32% (skin tests) and 46% (IgE assay) of mosquito bite test-positive individuals. Recombinant Aed a 3 has identical biological activity to its native form in mosquito saliva and can potentially be used in *in vivo* and *in vitro* tests and in immunotherapy for people with mosquito allergy.

Acknowledgments

We acknowledge Ms. Jieti Sun for her laboratory technical contributions to the study and the University of Minnesota Supercomputing Institute for providing all the necessary computational resources.

Author contributions

Z.P., W.W.X., Y.S., H.L., D.S., L.C., N.F.R., Q.G., and F.E.R.S. substantially contributed to conception and design of the study, acquisition of data, or analysis and interpretation of data. Z.P., W.W.X., A.A.J., and F.E.R.S. drafted the article or revised it critically for important intellectual content. Z.P. guarantees final approval of the version to be published.

Funding

This work was supported by grants from the Children's Hospital Research Institute of Manitoba (CHRIM) in Canada and conducted using facilities of the CHRIM. A.A.J. was supported in part by a grant from the National Institutes of Health in the USA (AI29746).

Conflict of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1 Supplementary data.

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