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

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Abstract

Allergic reactions to mosquito bites are an increasing clinical concern. Due to lack of availability of mosquito salivary allergens, they are under-diagnosed. Here, we reported a newly cloned mosquito *Aedes (Ae.) aegypti* salivary allergen and its clinical relevance.

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 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* mosquito bite tests were performed in 43 volunteers, some of whom had serum rAed a 3-specific IgE levels measured.

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 addition of natural mosquito extract dose-dependently. Immediate skin test reactions to rAed a 3 correlated significantly with reactions produced by mosquito bites. Of the bite-test positive volunteers, 32% had a positive rAed a 3 skin test and 42% 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.

Aed a 3 is a major mosquito salivary allergen. Its recombinant form has biological activity and is suitable for use in skin tests and serum IgE assays in mosquito-allergic individuals.

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The authors have declared that no competing interests exist.

Author Contributions

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Keywords

Aed a 3; *Aedes aegypti*; Insect allergy; Mosquito; Salivary allergen/antigen; allergens and epitopes; dermatology; IgE; immunologic tests; venom

Introduction

Mosquitos are a global problem, not only because they facilitate 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 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

Allergic reactions to mosquito bites are caused by salivary proteins introduced into the skin when adult female mosquitos probe and ingest a blood meal. 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 of mosquito allergy. We compared the UniCAP assay that utilizes mosquito whole-body extract with our mosquito saliva-capture enzyme-linked immunosorbent assay (ELISA) for measurement of mosquito-specific IgE. The results indicated that the mosquito saliva-capture ELISA is significantly more sensitive and specific (less likely to give false-negative and false-positive results) 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 Development of recombinant mosquito salivary proteins circumvents the problems involved in collecting mosquito saliva, potentially facilitating accurate and safe clinical evaluation and immunotherapy in mosquito-allergic individuals.

Aedes (Ae.) aegypti is an important mosquito species with a worldwide cosmopolitan distribution. The saliva of an adult female *Ae. aegypti* contains a complex of proteins in which at least 8 are allergens.¹⁸ We have previously expressed, purified, and clinically evaluated two recombinant *Ae. aegypti* mosquito salivary proteins^{19, 20} from two cDNAs previously isolated.^{21, 22} 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 1¹⁹ and the 37 kDa protein (biochemical name “D7”) is called Aed a 2.²⁰

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, designated Aed a 3, will facilitate studies of mosquito allergy and the mechanisms of pathogen transmission through mosquito saliva.

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 female and 21 male), ages 20 to 54 with a history of skin reactions to mosquito bites that ranged from highly sensitive to no reaction were selected 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; and 23 also had a 10 ml blood sample taken for assay of rAed a 3-specific IgE.

Mosquitos, mosquito antigens, and anti-saliva 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.^{18, 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 λ gt11 cDNA library of adult female mosquito *Ae. aegypti* (provided by Dr. Anthony James, University of California, Irvine, CA) was screened and two sets of PCR primer pairs were designed to combine two overlapping cDNA clones into a full-length Aed a 3 cDNA clone (Supplementary Data). 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-terminus sequence of Aeda3 was carried out using the Schrodinger modeling package (Schrodinger, LLC, New York, NY). The X-ray crystallographic structure of Anopheles anti-platelet 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-terminus sequence of Aed a 3 187-253. All missing sidechains 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 (Supplementary Data). Concentrated rAed a 3 supernatant was loaded onto a DEAE-Sephacel column and then eluted with a linear NaCl gradient (0 to 0.6 M in Tris buffer). Protein concentrations were monitored by OD₅₉₅ nm and rAed a 3 content assayed using ELISA and immunoblots (Supplementary data). 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 methods reported previously (Supplementary Data). These clone-specific antibodies were used in immunoblot analyses to detect their binding to the native protein present in the saliva of *Ae. aegypti*.

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, transferred to nitrocellulose membranes and immunoblotting analyses performed as described previously.¹⁸ Membranes were incubated with fusion protein-selected mouse antibodies or mouse anti-saliva 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) and then with ECL detecting reagents (Amersham Life Science, Buckinghamshire, England). 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), 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 Grove, PA). After washing, the color was developed by incubation of the membranes with an enzyme 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.^{20, 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 (1:20 diluted). The bound serum Aed a 3-specific IgE was detected by incubation of the plates with goat anti-human IgE, followed by incubations with alkaline

phosphatase-conjugated rabbit anti-goat IgG and finally with the enzyme 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 was diluted 10-fold. Each dilution was mixed with an equal volume of a 1:25 diluted mosquito-allergic serum. Serum diluted with PBST served as a positive 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 was traced with pen after 10 minutes and the other test sites were traced after 20 minutes and at 24 hours post-test. All tracings were transferred to paper and the areas measured.²⁷ An immediate wheal or a delayed induration of $\geq 0.3 \text{ cm}^2$ 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 regressions were used to determine correlations. All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Prism, USA). 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 anti-saliva serum. Three clones with different sizes of cDNA inserts were subcloned. One clone (λ AA22) 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. The primary nucleotide sequence of the Aed a 3 cDNA and its deduced amino acid sequence are described in Supplementary data and the sequence data were deposited in GenBank (AF001927).

Molecular antigen binding site analysis

The x-ray-resolved crystallographic structure of a mosquito (*Anopheles*) anti-platelet 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) (Figure 1A). The structure of the apap fragment is resolved in complex with the mouse Fab antibody at 1.8 Å 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. Examination of the antigen binding site with the nearest apap residues identifies the KDKNN sequence motif located at the inter-helical turn as the antigenic epitope site for antigen binding (Figure 1B). Based on these observations, homology modeling of the C-terminal region of Aed a 3 was carried out. The final model showed all four cysteine residues essential for the formation of the two disulfide bridges in apap are conserved within the Aed a 3 C-terminus region (Figure 1C). There is >30% reduction in the number of ionizable residues located on the surface of Aed a 3 C-terminus region as compared to apap, two-thirds of which comprise primarily lysine residues (Figure 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 (Figure 2, lane 2). This indicates that the cDNA that 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 Figure 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 (Figure 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.

Skin testing and clinical relevance

No systemic reactions were noted after the mosquito bite tests or skin tests. Twenty-eight out of the 43 participants (65%) had a positive immediate skin reaction in the bite test, while 15 (35%) had a negative bite test. Among the 28 bite-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) (Figure 5).

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

positive group than in the bite-negative group for both cut-off levels: 50% vs 13% in 1SD cut-off and 32% vs 0% in 2SD cut-off. Using the method reported²⁸ diagnostic values of rAed a 3-induced immediate reactions were calculated as “137” and “132”, respectively. Both cut-off 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-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 bite test-negative group ($P=0.0014$) (Figure 7). Using the geometric mean of the controls plus 2 SD as a cut-off level, ~46% (6/13) mosquito bite-positive individuals exhibited a positive serum rAed a 3-specific IgE, while none of the bite-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 I).

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 dodecylsulphate polyacrylamide gel electrophoresis;²⁹ in 2007 it was reported to contain 55 proteins coded by salivary gland-specific transcripts.³⁰ Among them, at least 8 proteins have been identified as allergens that bind to the IgE of allergic individuals who have large local (skin) reactions to mosquito bites.¹⁸ 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 for IgE binding at the Aed a 3 C terminus 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 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.

Taken together, the above evidence indicates that Aed a 3 is an IgE-binding allergen and that rAed a 3 expressed using a baculovirus/insect cell system has identical antigenicity and biological activity to the natural form of Aed a 3 in *Ae. aegypti* saliva. Because rAed a 3 was expressed using a baculovirus/insect cell system that performs many of the post-translational modifications found in mammalian cells,³¹ it is not surprising that the rAed a 3 we produced has biological activity and the potential to be developed as a novel allergen.

Molecular cloning facilitates isolation and unlimited production of purified recombinant proteins. These 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 was proved to be an excellent candidate for use in in vivo and in vitro tests with 100% specificity in mosquito bite-negative individuals for the diagnosis of mosquito allergy. We have previously expressed and clinically evaluated 2 recombinant *Ae. aegypti* salivary allergens, rAed a 1¹⁹ and rAed a 2²⁰ in addition to rAed a 3. Preparation of additional recombinant mosquito salivary allergens is in progress. Establishment of assays using a cocktail or 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 after an 18-month course of injections, skin reactions and respiratory and eye symptoms after mosquito bites were reduced.³⁵ Application of recombinant allergens or their modified forms will potentially enhance the effectiveness and safety of this specific immunotherapy. As the binding sites of this molecule have been identified to locate 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 cut off level of the mean of controls plus 2SD, rAed a 1 induced a positive skin reaction in 29%¹⁹ or 43%^{37, 38} of the mosquito bite-test positive group and 0 in the bite-negative group, while rAed a 2 induces a positive reaction in 11% of the bite-positive group and 0 in the controls.^{37, 38} In another study of rAed a 2,²⁰ using mean plus 1SD of control group as a cut-off 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 different participants, allergen batches and cut-off levels selected. In the present study, even at a cut-off 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-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 (GenBank #AF001927, deposited in 1997),³⁹ a study showed that the Aed a 3 coding protein, the Aegyptin protein (the biochemical name of Aed a 3, encoded by the same gene), secreted from the female *Ae. aegypti* salivary glands, plays a critical, non-redundant role in the feeding success of this mosquito on vertebrate hosts.^{40, 41} This protein 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 be used in in vivo and in vitro tests and in immunotherapy for people sensitized to mosquito saliva. In addition, Aed a 3 protein is involved in *Ae. aegypti* feeding success, an aspect that deserves to be investigated further, as *Ae. aegypti* is the main vector of dengue, chikungunya, and yellow fever viruses.^{42–44}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

apap	anti-platelet aggregation protein
Ae.	<i>Aedes</i>
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	<i>Spodoptera frugiperda</i> f9 cells

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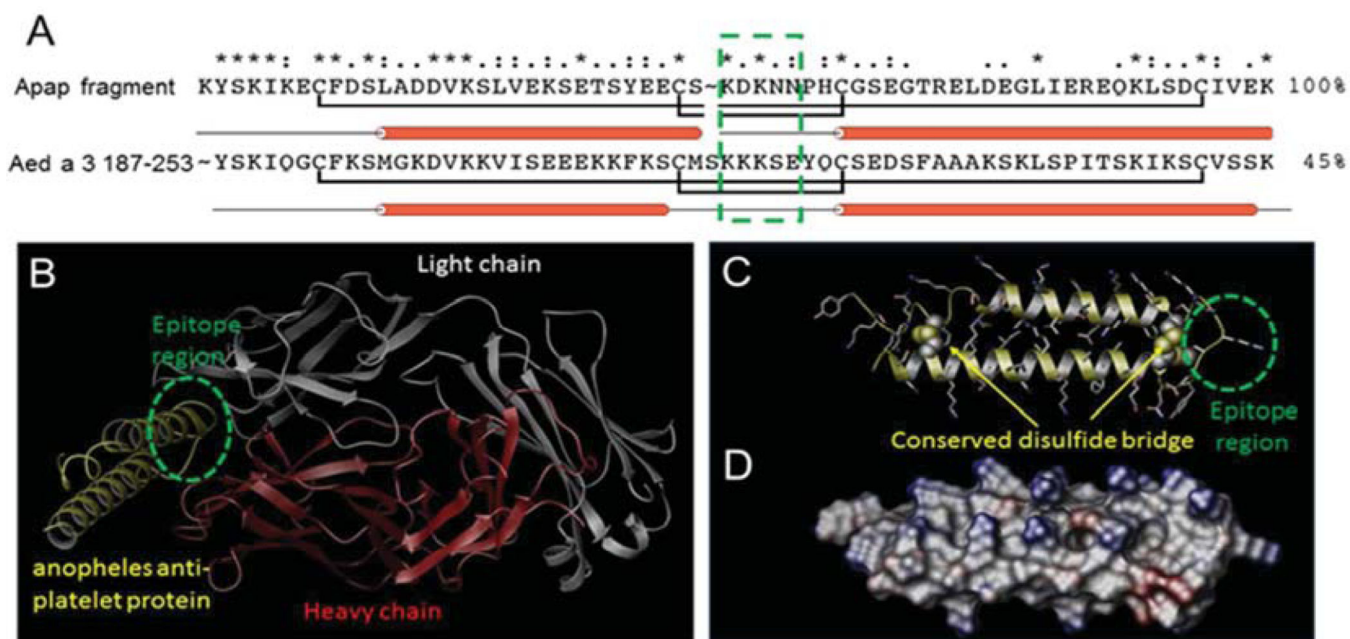


Figure 1. Homology model of the Aed a 3 C-terminal region. (A) Sequence alignment between the apap fragment and Aed a 3 C-terminal region. The two conserved disulfide bridges are highlighted with the antigenic epitope region boxed in green. (B) X-ray structure of apap fragment (yellow) in complex with mouse FAB antibody consisting of a light chain (white) and a heavy chain (red). (C) Homology model of the Aed3 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.

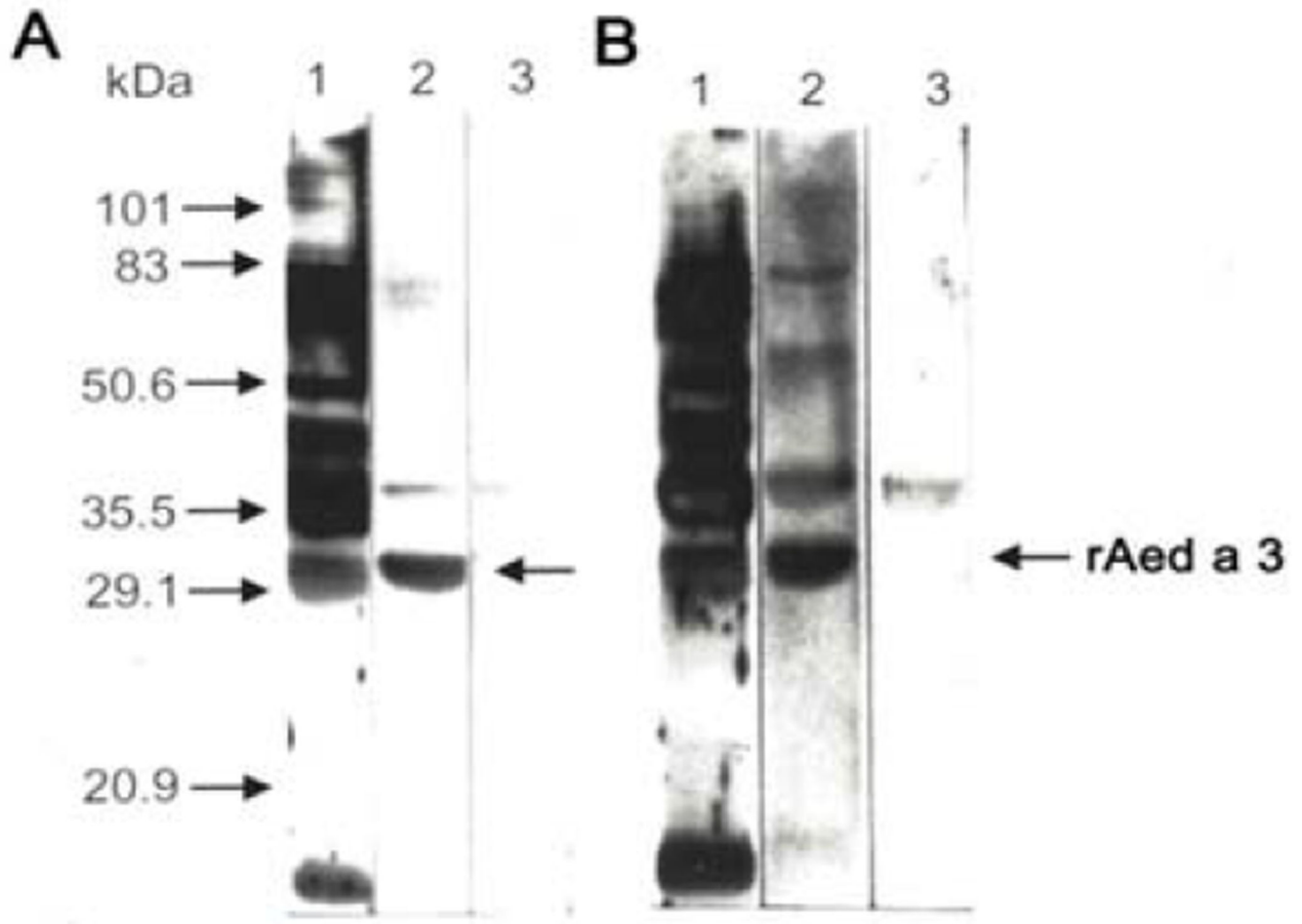


Figure 2.

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 anti-saliva 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.

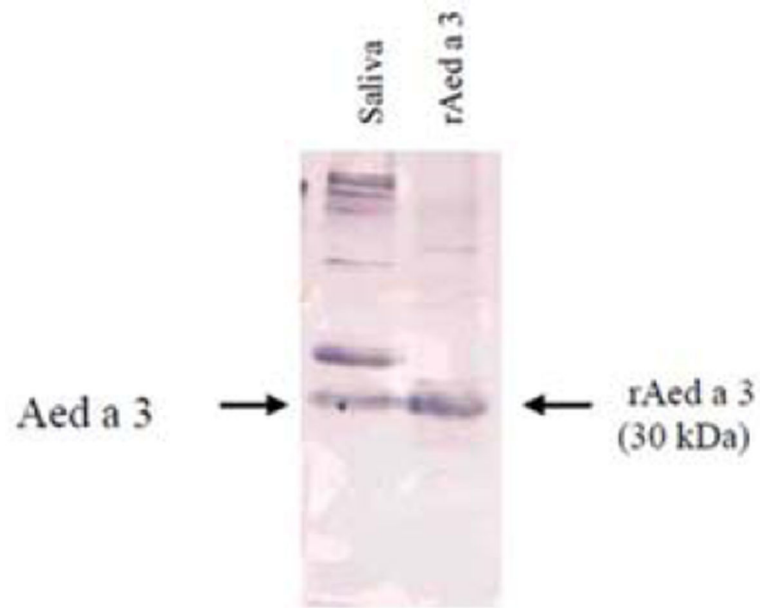


Figure 3. rAed a 3 protein binds to the IgE in mosquito-allergic human serum. Proteins in *Ae. aegypti* saliva (lane 1) and purified rAed a 3 (lane 2) separated by 10% SDS-PAGE were transferred to nitrocellulose membrane. The membrane was immunoblotted with a pooled mosquito-allergic human serum and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

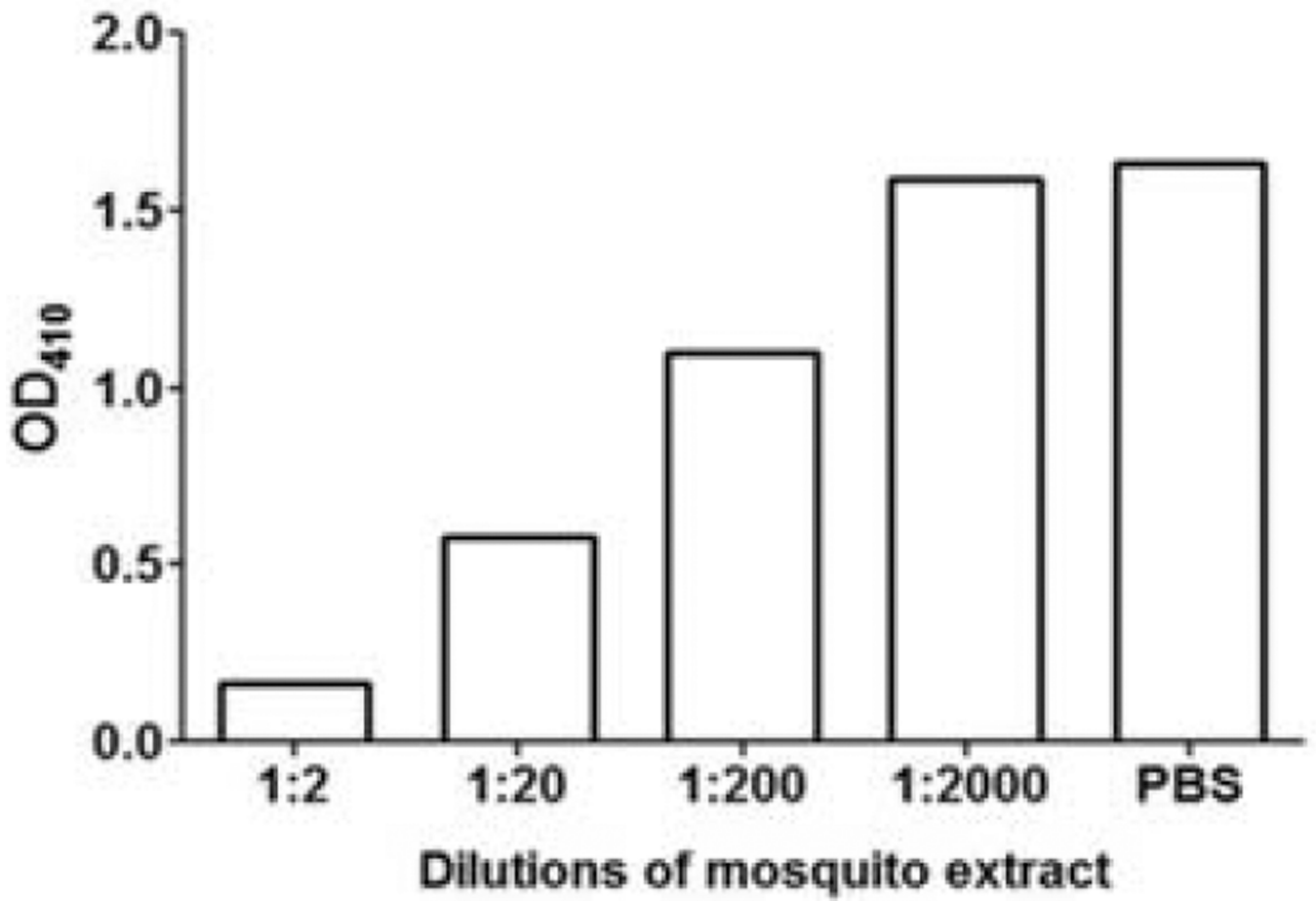


Figure 4.
Binding of rAed a 3 to human IgE can be inhibited in a dose-dependent manner by a natural *Ae. aegypti* head and thorax extract.

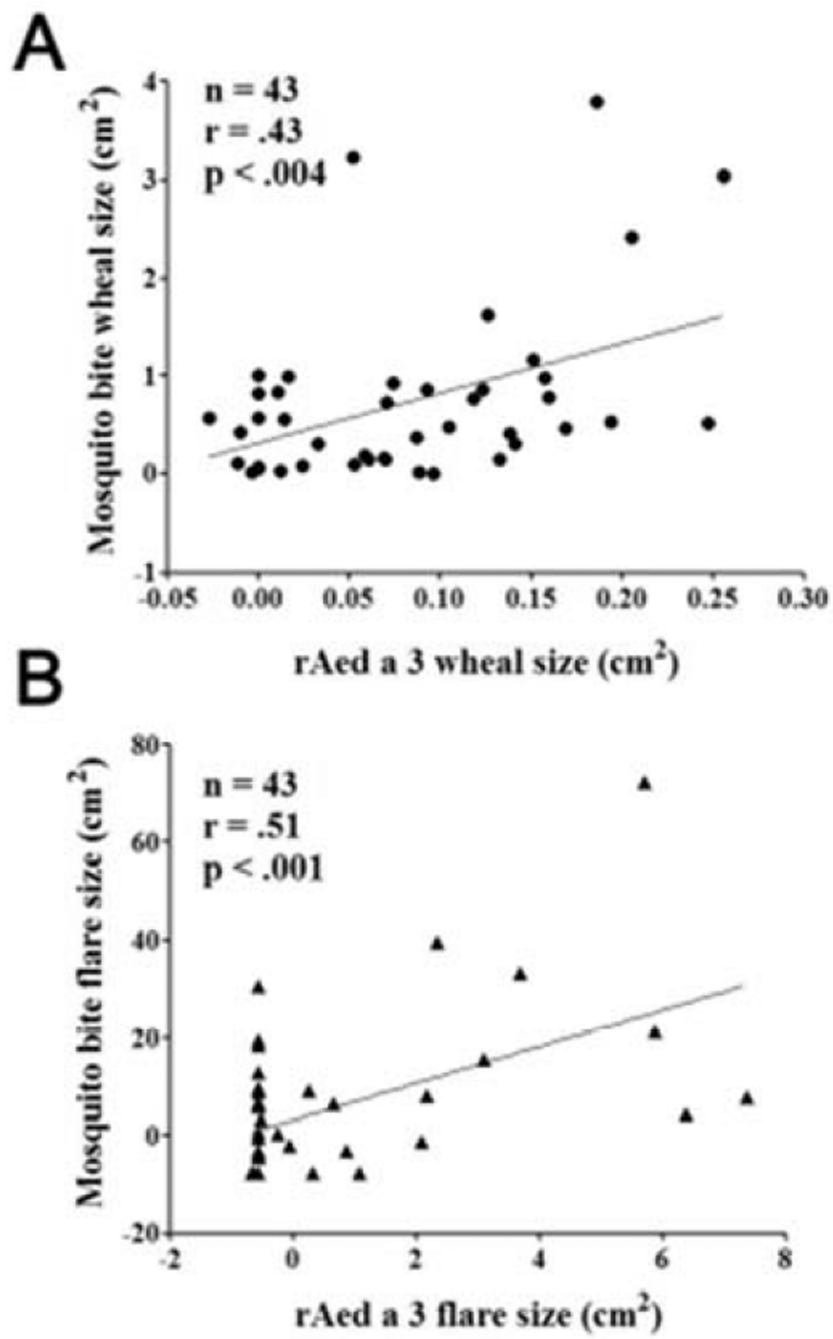


Figure 5. rAed a 3-induced skin flare and wheal sizes correlate significantly with those induced by mosquito bite tests.

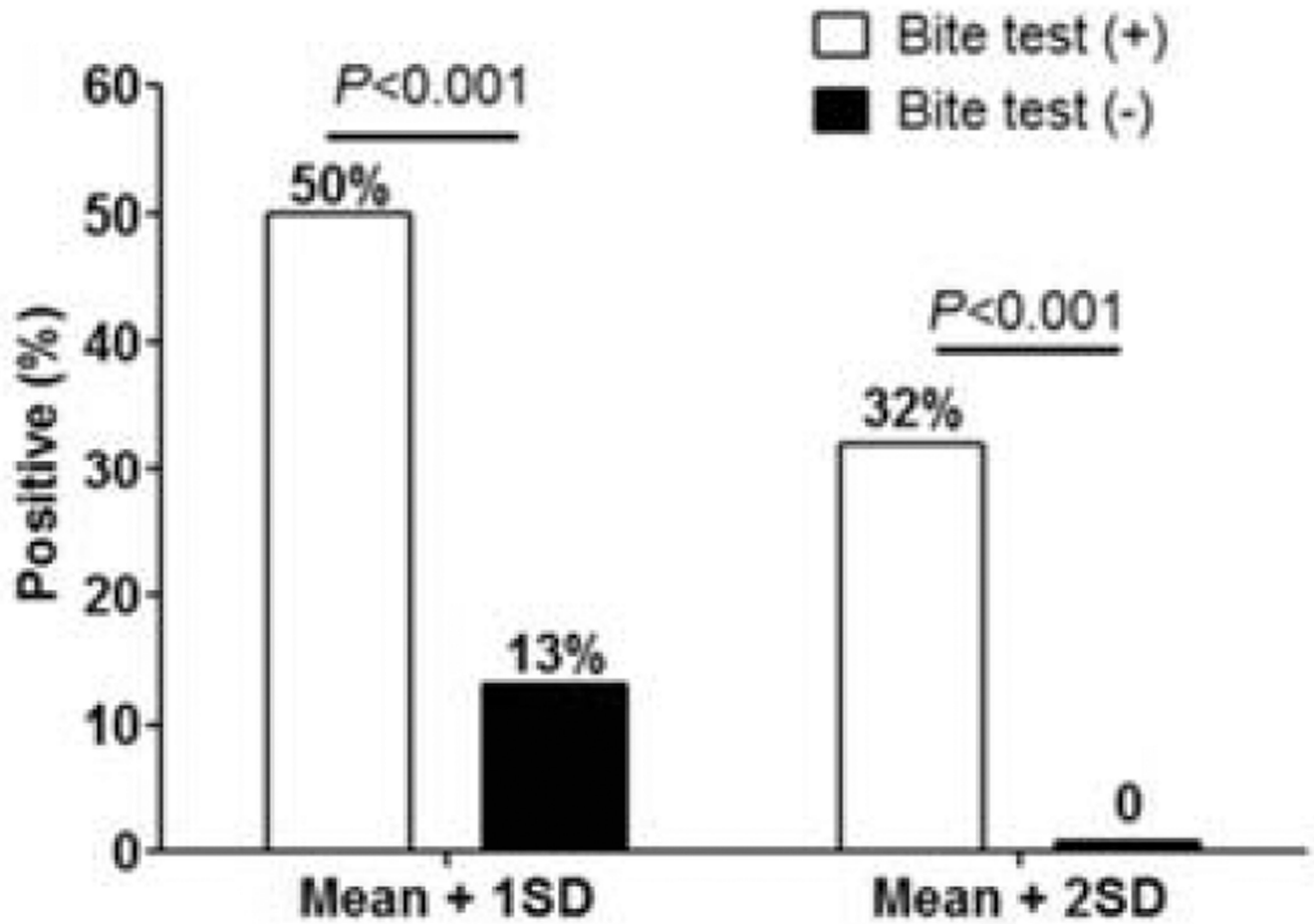


Figure 6. The positivity rate of skin prick testing with rAed a 3 is significantly higher in the *Ae. aegypti* bite-positive group (n=28) than the negative group (n=15). Two positive cut-off levels: the mean of rAed 3-induced skin positive reactions in the bite-negative group plus 1SD or 2SD.

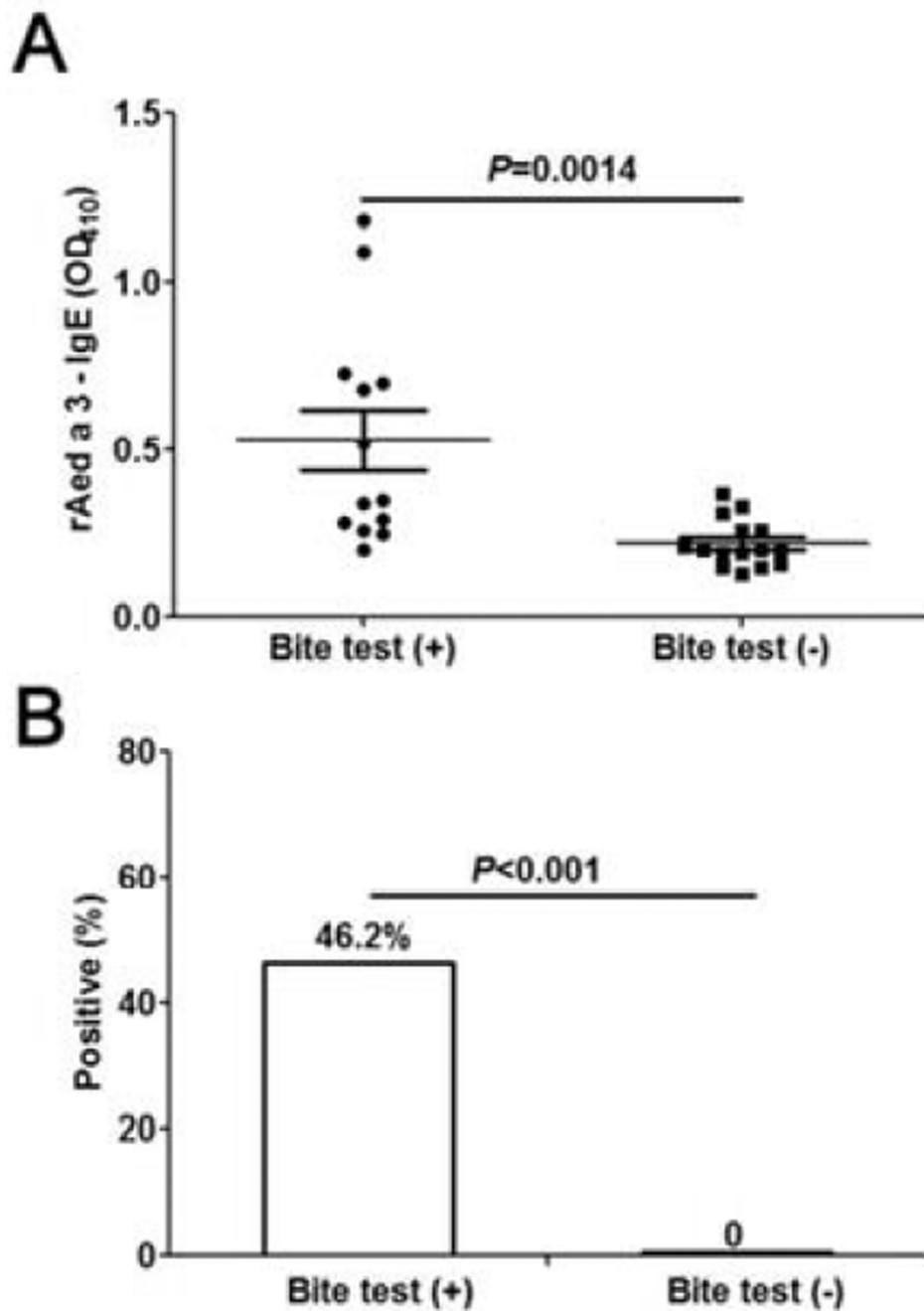


Figure 7. Serum rAed a 3-specific IgE levels are elevated significantly in the *Ae. aegypti*-bite test-positive group (n=13) compared with the bite-negative group (n=15). The geometric mean of the negative controls plus 2 SD was used as a cut-off level.

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, its diagnostic value, is the sum of its sensitivity and its specificity. It ranges from “failure” at “0” to “perfect” at 200%.

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