

Humoral immune responses in mice to a  
mosquito salivary gland antigen as a  
target for a diagnostic and  
immunotherapeutic agent

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Directed by Professor Dong Soo Kim

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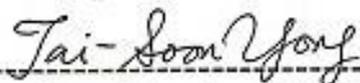
Kyong Min Choi

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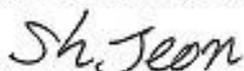
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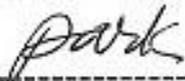
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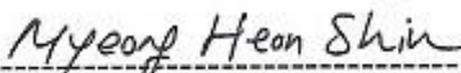
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Kyong Min Choi

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## ABSTRACT

Humoral immune responses in mice to a mosquito salivary gland antigen  
as a target for a diagnostic and immunotherapeutic agent

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(Directed by Professor Dong Soo Kim)

The mouthparts of mosquito are modified to pierce the tissues and suck blood from humans or other mammals. Their bites can cause immediate cutaneous reactions, such as wheal and flare, delayed reactions and occasionally systemic reactions in humans. IgE-mediated allergic reactions caused by mosquito bites are a common problem all over the world. This study was undertaken to determine IgE levels in anti-mouse serum, to elucidate mouse IgE binding patterns and to investigate the immunogenicity of salivary gland antigens of *Aedes togoi*. Mosquito larvae of *Aedes togoi* were collected and maintained in the laboratory. The mosquito specific mouse IgE level was measured using ELISA. Polypeptide patterns were analyzed by SDS-PAGE. Western blot was performed with sensitized immune mouse sera, and elucidated mouse IgE binding patterns to salivary gland extracts.

Protein band patterns of the salivary gland extracts (SGE) and whole body extracts (WBE) of the specimens were different from one another. Specific mouse IgE reacted to the protein in SGE of 18.0, 33.0, 35.0, 37.0, 45.0, 57.5, 72.0, 90.0 and 150.0 kDa from *Aedes togoi*. Molecular biological techniques were used to study the genetic information and functions of genes. The cDNA sequencing was carried out to elucidate and compare the genome of *Aedes togoi* with other coded gene of the allergen. Two previously unknown protein coding genes(DUF 1398, DUF 2528) were identified among the 45 positive clones prepared from the mosquito salivary gland by immunoscreening. Analysis of the 3D structure of DUF1398 and DUF2528 was not similar with any other allergens identified in plants or animals, despite low sequence identities to their templates, the global folds of the 3D models of the cockroach allergen *Bla g 4* and the mosquito salivary protein antigen *Aed a 2* had a sizable fraction of structural overlap, suggesting that it would be a potential target for therapeutic agents in specific mosquitoes bite allergens.

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Key words : allergen, mosquito, *Aedes togoi*, salivary gland, IgE

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## **I. INTRODUCTION**

Mosquitoes transmit infectious diseases, such as malaria, filariasis, Japanese encephalitis B. The life cycle of the mosquito is a complete metamorphosis; the egg, larva, and pupa reside in water. Only female mosquitoes suck blood and transmit pathogens. Three mosquito genera are of medical importance<sup>1</sup>. They are *Anopheles*, *Culex*, and *Aedes*. They differ in distribution, morphology, ecology, and diseases transmission (Fig. 1).

During an insect bite, the salivary glands release components that include antihistamines, vasodilators like tachykinin, anticoagulants like thrombin and factor IXa-directed molecules<sup>2</sup> and immunomodulators, in order to facilitate entry of inoculum containing pathogens. The salivary components of vectors have been implicated to be of importance in transmission of pathogens (viral,

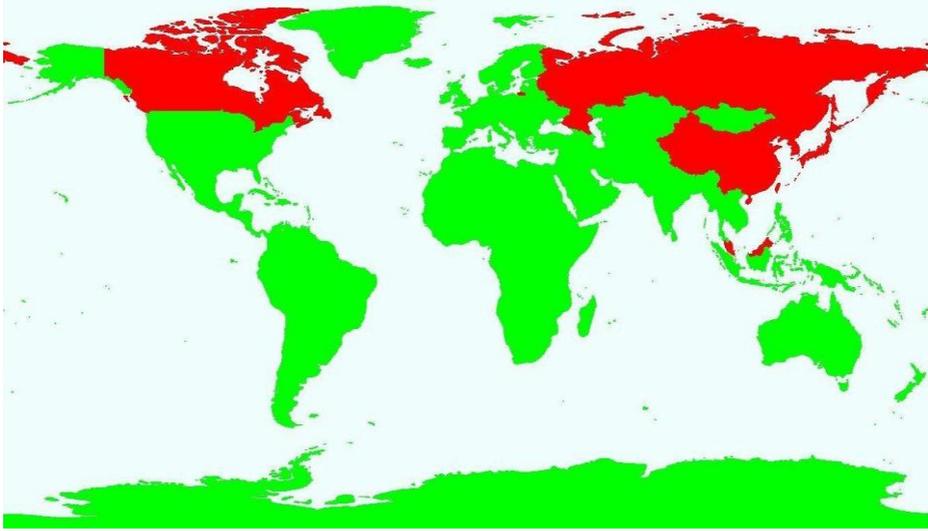


Figure 1. Distribution of *Aedes togoi*. This map denotes only the country or general areas where *Aedes togoi* (red areas) has been recorded, not actual distribution. (© 2006 M. Disbury SMS-NZB [www.smsl.co.nz](http://www.smsl.co.nz))

bacterial and protozoan) by ticks and mosquitoes<sup>3</sup>. Salivary glands of other blood-sucking arthropods like a star tick bear prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) receptor that stimulates secretion of an anticoagulant in order to facilitate blood feeding<sup>4</sup>.

Reactions to mosquito bites are immunologic in nature. They are due to specific sensitization to the mosquito salivary proteins, because initial exposure to mosquito species to which an individual has not been previously exposed causes no reaction. Mosquito bite-induced immediate wheals and flares reaction correlate well with mosquito salivary gland-specific IgE levels. The development of skin sensitization to mosquito bites also parallels the levels of saliva-specific IgE antibodies. Mosquito saliva-specific IgG

antibodies, consisting mainly of the IgG1 and IgG4 subclasses, have been found to be significantly elevated in individuals with positive mosquito bite tests and in individuals with severe local reactions, but not systemic reactions, to mosquito bites<sup>5</sup>.

IgE-mediated allergic reactions caused by mosquito bites are a common problem all over the world. Mosquitoes inject saliva containing antigenic proteins into the bite wound<sup>6,7</sup>. Their bites can cause immediate cutaneous reactions, such as wheal and flare, delayed reactions and occasionally systemic reactions in humans<sup>8,9</sup>.

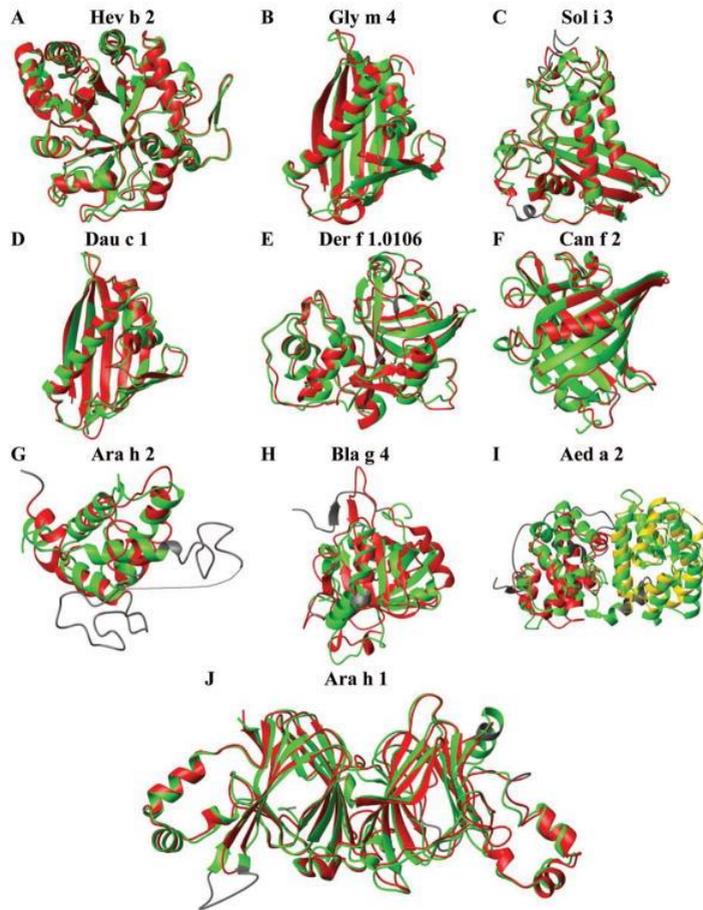
It has been reported that mosquito saliva-specific IgE and IgG antibodies are involved in mosquito bite allergy<sup>10</sup>. In order to confirm these responses, enzyme-linked immunosorbent assay (ELISA)<sup>11</sup> and immunoblot techniques<sup>12</sup> have been carried out. *Aedes togoi* transmit infectious organisms including filaria. The *Aedes togoi* is distributed on most islands and at the seaside in Korea. However, the allergenicity of saliva antigen in humans and experimental mice has not been reported until now. The saliva allergen was prepared from salivary gland extracts and they probably mixed a large admixture of protein. Mosquito larvae of *Aedes togoi* was collected and maintained in the laboratory. Salivary gland extracts (SGE) and whole body extracts (WBE) were prepared from female mosquitoes.

This study was undertaken to determine the IgE levels in anti-mouse serum, to elucidate mouse IgE binding patterns and to investigate the reactivity of salivary gland antigens of *Aedes togoi*. The mosquito specific mouse IgE level was measured by using ELISA. Polypeptide patterns were analyzed by SDS-PAGE. Western blot was performed with sensitized immune mouse sera, and elucidated mouse IgE binding patterns to SGE. Protein band patterns of the SGE and WBE of the specimens were different from one another. The molecular biological techniques were used to study the genetic information and functions of genes in a certain individual organism. The

number of important allergens has been expressed by using recombinant DNA technology<sup>13</sup>. The cDNA sequencing has been carried out to elucidate *Aedes togoi* coded gene of the allergen. Utilization of this technique to produce pure mosquito allergens in large quantities would be an important advance in immunology.

The three-dimensional (3D) structures of macromolecules, as collected and provided by the Protein Data Bank (PDB), offer tremendous insights into the molecular function at the atomic level, and they often provide direct evidence for aspects of that function by exemplifying molecular interactions between individual macromolecules, or between macromolecules and small molecules<sup>14</sup>. We evaluated the similarities of the 3D folds, and found two novel proteins. Our results from this study indicate that the examined models in two novel proteins are useful for determining other characteristics of the allergenic proteins.

Structural similes of allergens are needed to supply a molecular interpretation for clinically observed cross-reactivities between allergens from different organisms<sup>15-25</sup> and to predict whether new proteins or other biotechnology products are potential allergens<sup>14,36</sup>. However, there are only 86 experimental 3D structures in the Protein Data Bank (PDB) for allergens<sup>14</sup>, a small fraction of the 1499 allergen and isoallergens sequences collected in the Structural Database of Allergenic Proteins (SDAP)<sup>26</sup>. Thus reliable, template-based models of allergens are needed to compare allergen features, and to determine potential cross-reactive IgE binding surfaces<sup>27</sup>.



*Proteins. 2013;81:545–54.*

Figure 2. Structural overlays of aligned regions of the structural database of allergenic proteins model structures (red, yellow) with the corresponding experimental structures (green). (A) 1,3-glucanase from latex rubber (Hev b 2), (B) Stress-induced protein SAM22 from soybean (Gly m 4), (C) Venom allergen III from fire ant (Sol i 3), (D) Pathogenesis-related protein PR-10 from carrot (Dau c 1), (E) Cysteine protease from American dust mite (Der f 1.0106), (F) Canine salivary lipocalin (Can f 2), (G) Conglutin from peanut (Ara h 2), (H) Calycin from German cockroach (Bla g 4), (I) Salivary odorant binding protein D7 from mosquito (Aed a 2), (J) Vicilin from peanut (Ara h 1).

## II. MATERIALS AND METHODS

### 1. Mosquitoes and salivary gland extracts

Mosquito larvae are common in the coastal regions, usually occurring in tidal pools or rock pools of saline or brackish water above the tidal zone, but also occasionally in containers such as used tires with fresh water<sup>28</sup>. Larvae of *Aedes togoi* were brought from rock pools at the seaside to the laboratory and reared in the cages (Fig. 3).



Figure 3. Collection of mosquito larvae. Mosquito larvae were collected from rock pools at the seaside at Song-jong Dong, Pusan for *Aedes togoi*.

The collected larvae were maintained for emergence in the laboratory and once the adults emerged, females were collected, anesthetized by CO<sub>2</sub> gas and 20,000 female adult *Aedes togoi* were taken for the study. Dissection of salivary gland tissues from mosquitoes requires prior preparation of 1X phosphate buffered saline (1X PBS) solution and anesthetization of mosquitoes by subjecting to a temperature of 4°C, until immobilized. The mosquitoes remain anesthetized by placing in a Petri dish that is kept cold on

ice. Other materials required include: light microscope fitted with 10x objective lens, pipette, fine-tipped forceps, glass slide, and needle-tip probes. Salivary glands were obtained by dissection of 3 to 5 day old adults in 0.02 M PBS, pH 7.2, under a stereomicroscope, and transferred to fresh PBS on ice (Fig. 4). About 100 pairs of salivary glands were obtained and lyophilized.

The salivary gland extracts were reconstituted by dissolving the lyophilized salivary gland proteins in PBS before use. This was centrifuged at 12,000 xg for 20 min. The supernatant was filtered through a 0.22  $\mu\text{m}$  Amicon filter and the protein concentrations were measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA).

## **2. Production of antisera in mice**

The mice were immunized by the method of Brummer-Korven H with some modifications<sup>29</sup>. Briefly, 20 live female *Aedes togoi* per BALB/c mouse were released to inject the antigens naturally, twice a week, for 12 weeks. Serum samples were collected once after 12 weeks the immunization period. All sera were stored at  $-80^{\circ}\text{C}$  until analysis.

## **3. Enzyme-linked immunosorbent assay**

The enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and color change to identify a substance. For the enzyme-linked immunosorbent assay, mosquito-specific IgE levels in mouse serum samples were first directly coated on 96-well polystyrene plate. Mosquito-specific IgE level in sensitized mouse serum sample was measured by ELISA. To perform the ELISA kit (BioLegend, Mouse IgE, CA, USA) (Fig. 5), dilute capture antibody in coating buffer (0.05 M carbonate buffer, pH 9.6). Add 100  $\mu\text{l}$  of this capture antibody solution to all wells of a 96-well plate provided in this set. Seal plate and incubate overnight (16-18 hrs) at  $4^{\circ}\text{C}$ . Wash plate 4 times with 300  $\mu\text{l}$  wash buffer (0.05% Tween 20 in 0.01 M PBS, pH 7.2) per well

and to block non-specific binding and reduce background, add 200  $\mu$ l 1X Assay Diluent A per well. Seal plate and incubate at RT for 1 hour with shaking at 200 rpm on a plate shaker. Prepare 1,000  $\mu$ L of top standard at 10 ng/ml in 1X Assay Diluent A (refer to Reagent Preparation). Perform six two-fold serial dilutions of the 10 ng/ml top standard with Assay Diluent A in separate tubes. After diluting, the mouse IgE standard concentrations are 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.313 ng/ml and 0.156 ng/ml, respectively. Assay Diluent A serves as the zero standard (0 pg/ml)

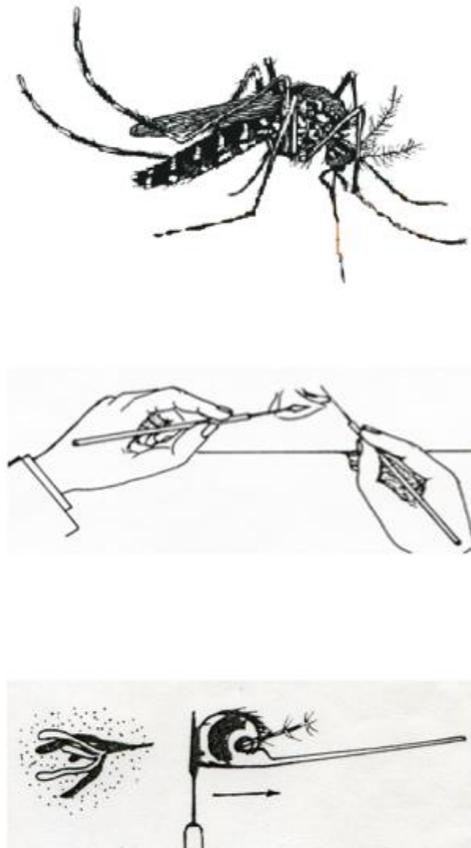


Figure 4. The isolation of salivary glands. Place a drop of 1X PBS onto a glass slide mounted under a light microscope. Pick up a mosquito by stabbing the thorax with a needle-tip probe. Pull off the mosquito legs using your fingers. Transfer the mosquito onto the slide. Remove the head of the mosquito using forceps. While holding down the mosquito thorax with the probe, use another probe to gently push down on the thorax. The salivary glands are located at the anterior portion of the thorax and can be isolated by using a needle-tip probe and severing the attachments that connect the gland to the thorax. Intact salivary glands are comprised of three lobes: two lateral lobes and a medial lobe.

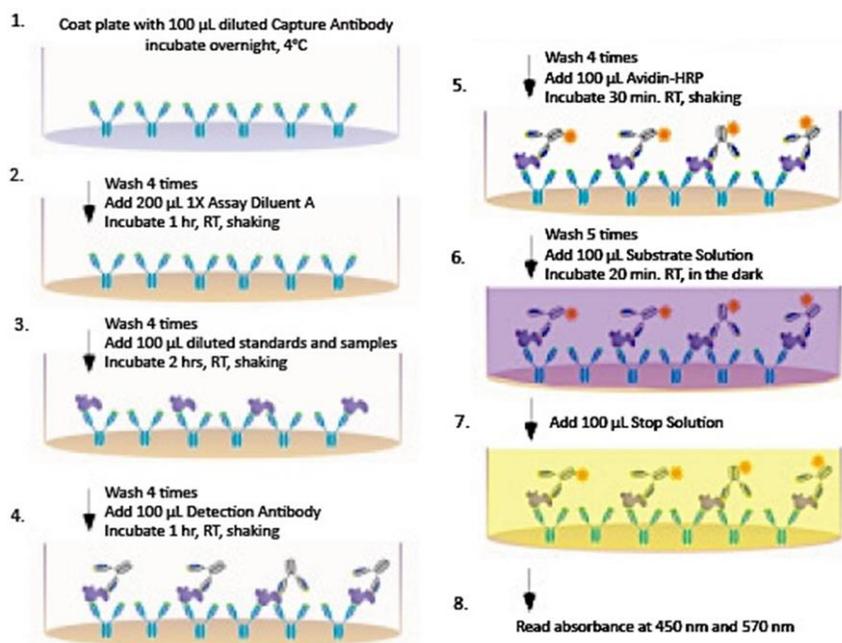


Figure 5. Summary of Assay Procedure. Wash plate 4 times with wash buffer and add 100  $\mu\text{L}$ /well of standard and samples to the wells. Seal plate and incubate at RT for 2 hours with shaking and then wash plate 4 times, add 100  $\mu\text{L}$  of diluted Detection Antibody solution to each well, seal plate and incubate at RT for 1 hour with shaking. Wash plate 4 times and add 100  $\mu\text{L}$  of diluted Avidin-HRP solution to each well. Seal plate and incubate at RT for 30 minutes with shaking. Wash plate 5 times with wash buffer, for this final wash, soak wells in wash buffer for 30 seconds to 1 minute for each wash. Add 100  $\mu\text{L}$  of freshly mixed TMB Substrate Solution and incubate in the dark for 20 minutes. Stop reaction by adding 100  $\mu\text{L}$  of stop solution (1 N NaOH) to each well. Read absorbance at 450 nm within 30 minutes (Dynatech, MR-5000, Chantilly, VA., USA). All serum samples were analyzed in duplicate.

#### **4. SDS-PAGE and Immunoblot Analyses**

SDS-PAGE was performed by the method of Laemmli with 12% acrylamide separating gels<sup>30</sup>. SGE of mosquitoes separated by SDS-PAGE were then electrophoretically transferred to a nitrocellulose membrane by the method of Towbin et al. Membranes were incubated with pooled serum samples with positive ELISA titers and sera of controls. Nitrocellulose membrane was blocked for overnight at 4°C with 3% BSA. After several washes for 45 min, the membrane was incubated with 1/10 diluted human pooled antisera overnight at 4°C, and then with HRP-conjugated anti-mouse IgG for 35 min at RT. After several washes for 45 min, immediately pour the chemiluminescence reagent into the weigh dish with the membrane and incubate for 1 min at room temperature. Use at least 1 mL per membrane and incubate. Take the blot to the developing room and place the membrane between the covers of a propylene sheet protector with the black interface removed (plastic wrap works well also). Switch off the lights and place the Kodak Scientific Imaging film on top of the membrane. Repeat the exposure, varying the time as needed for optimal detection.

#### **5. Immunohistochemistry and confocal imaging for analysis of fine structure of salivary glands**

To identify expression profiles, the salivary glands were analyzed for fine structure *in vivo*. Adult females were used for the tissue specimens. The tissue specimens were fixed in 3.7% PFA for 1 day at 4°C and decalcified with 10% ethylene di-amine tetra-acetic acid for 2 days. Making paraffin block and then, 4 µm-thick serial sections were cut and processed for hematoxylin and eosin staining. For fluorescence immunohistochemical staining, the tissue sections were deparaffinized and hydrated with alcohol series. After antigen retrieval in citrate buffer, the sections were treated with hydrogen peroxide.

After blocking with 3% BSA in PBS overnight at 4°C, the whole-mounted specimens were incubated for 2 hrs at RT with sensitized mouse antibodies and then incubated for 1 hr at RT with secondary antibodies. Salivary gland proteins were detected as a red color using anti-mouse-Rhodamine Red™. For cytoskeleton, Phalloidin was detected as a green color using anti-Phalloidin-fluorescein isothiocyanate (v/v, 1:1000, Molecular Probes). The nuclei were stained in 4',6-diamidino-2-phenylindole (DAPI, 2 µg/ml) for 5 min at RT. Specimens were imaged by a LSM 510 META confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

## **6. Bacterial strains and phage**

The *E. coli* strains used in this study are XL1-Blue MRF' and SOLR. The characteristics of these microorganisms were described in Table 1. For Mass-clone excision, f1 helper phage was used at the concentration of  $1.2 \times 10^9$  pfu/µl. The Uni-ZAP XR (Stratagene, CA, USA) was used for the construction of cDNA library (Fig. 6).

## **7. Total RNA and Poly (A)+ RNA**

Total RNA was isolated from 1g of 5,000 females of *Aedes togoi* using Trizol reagent (Invitrogen, CA, USA). One microgram of frozen salivary gland was incubated in 2ml Trizol reagent for 5 min at RT to permit the complete dissociation of nucleoprotein complexes. After mixing, 400ul chloroform was added and vortexed for 15 sec. The sample was centrifuged at 12,000xg for 15 min at 4°C. The supernatant was transferred to a new tube and mixed with 5 ml isopropyl alcohol and then incubated at RT for 10 min. After centrifugation at 12,000xg for 10 min at 4°C, RNA pellet was resuspended in 1 ml of 0.1% RNase free water. Poly (A)+ RNA was isolated from 0.03mg of the total RNA using oligo (dT) cellulose column. The quality and quantity of RNA were

checked by UV spectrophotometer (OD 260/280).

Table 1. The Character of *E. coli* strains used in this study.

Host	Genotypes
XL1-Blue MRF <sup>'</sup>	$\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 \quad endA1$ <i>supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB</i> <i>lacI<sup>q</sup>ZAM15 Tn10 (Tet<sup>r</sup>)]</i>
SOLR	e14-(McrA-) $\Delta(mcrCB-hsdSMR-mrr)171 sbcC recB$ <i>recJ uvrC umuC::Tn5 (Kan<sup>r</sup>) lac gyrA96 relA1 thi-1</i> <i>endA1 <math>\lambda^R [F' proAB lacI<sup>q</sup>ZAM15]^C</math> Su-</i>

## 8. Construction of cDNA library

The cDNA library was constructed from mRNA using a ZAP-cDNA synthesis kit (Stratagene, USA). Five micrograms of poly(A)+ RNAs were primed with a 50-base oligo (dT) primer containing Xho I site at the 3'-end. First strand cDNA was synthesized with the linker-primer (5'-GAG AGA GAG AGA GAG AGA GAA CTA GTC TCG AGT TTT TTT TTT TTT TTT TTT TTT-3') and is transcribed using Maloney Murine Leukemia Virus – Reverse Transcriptase (MMLV – RT) and 5-methyl dCTP to create hemimethylated cDNA. RNase H and *E. coli* DNA polymerase synthesized double-stranded cDNA. After ligation of EcoRI adapter, and phosphorylating the EcoRI ends onto the cDNA, it was fractionated through sepharose CL-2B gel filtration medium in drip column. After ligated cDNA into the Uni-ZAP XR vector (Fig. 6), it was packaged in vitro using Gigapack III Gold kit. After titering, the primary library were amplified and stored prior be used.

## 9. Immunoscreening of the cDNA library and characterization of selected immunoreactive clones

The Uni-ZAPTM premade ovarian carcinoma cDNA expression library was purchased from Stratagene, La Jolla, CA, USA. cDNA expression library of the mosquito MSQ452 and MSQ49 were screened with sensitized mouse serum. The cDNA library was plated on NZY agar plates at a density of 400 clones/10 cm plate. The plates were incubated at 42°C for 4 hrs to allow plaques to develop. Nitrocellulose filters soaked with isopropyl β-D-thiogalactopyranoside (IPTG) were then laid on top of the plaques and incubated at 37°C for 4 hrs to transfer the plaques onto the membranes. Subsequently, the filters were blocked with 1% acetone powder solution/PBS-T (80 mM sodium orthophosphate, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, and 0.1%

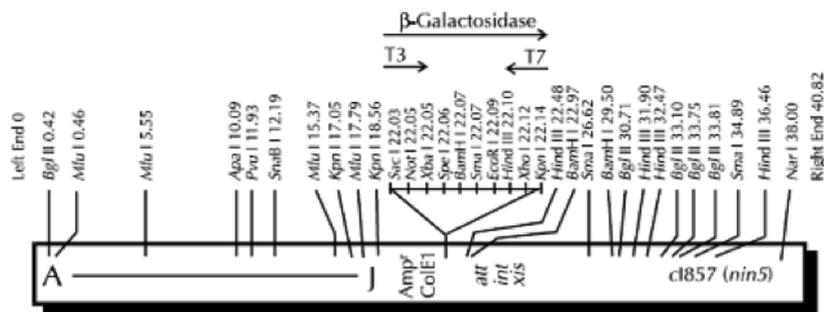


Figure 6. Lambda Uni-Zap XR insertion vector.

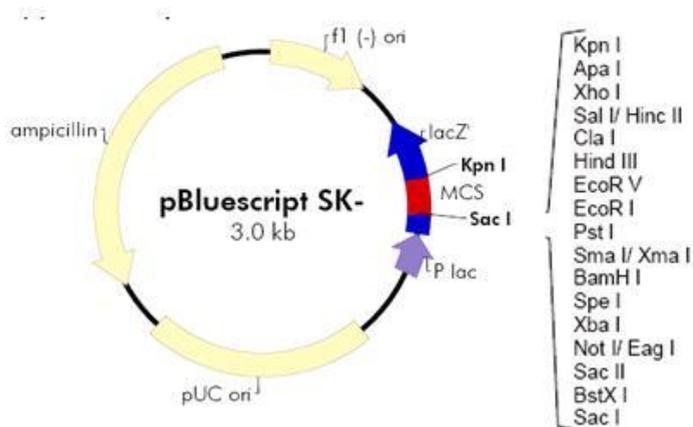


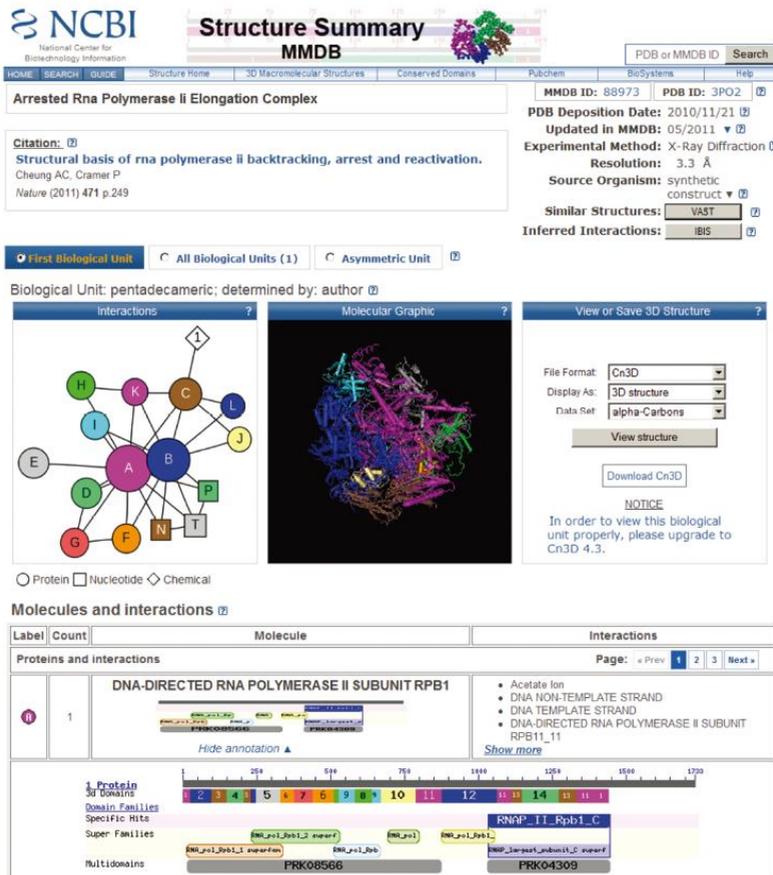
Figure 7. Cloning plasmid for genomic inserts. Lambda ZAP carries pBluescript (-), which is excised in vivo upon infection with f1 or M13 helper phages. Inserts are cloned within a polyliner located within lacZ. As with  $\lambda$ gt11, a fusion protein may be expressed if the insert DNA is in frame with the lacZ sequence; thus, libraries made in this vector can be screened with antibodies. In  $\lambda$ ZAP, T7 and T3 promoters flank the inserts, which allow RNA probes to be easily obtained. pBluescript M13 (-), the excised plasmid is normally propagated as a double-stranded circular DNA, but infection with a helper phage enables the plasmid to be propagated as single-stranded DNA. DNA fragments up to 10 kb can be inserted. Within the polylinker, unique *Xho* I and *EcoR* I cloning sites are available.

Tween-20) overnight, at 4°C. To screen the library, the preabsorbed sensitized mouse serum 1:200 in 5% bovine serum albumin/PBS-T. The diluted sensitized mouse serum was first incubated with *E. coli* phage lysate (Stratagene, USA) for 2 hrs at room temperature to minimize the cross-reaction between the autoantibodies and the bacterial/phage proteins. The screening procedure was followed as described in the instruction manual (Stratagene, CA, USA). In brief, serum samples diluted 1:10 were preabsorbed with lysate from *E. coli* and bacteriophage-infected coupled to sepharose 4B (BioDynamics Lab Inc., Tokyo, Japan). Nitrocellulose filters were then incubated with this preabsorbed lysate from *E. coli* for 2 hrs at room temperature to identify cellular proteins that react with the autoantibodies in the mouse serum. Following probing with the sensitized mouse serum, the filters were washed with PBST for five times and further treated with alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) diluted 2000-fold in 5% bovine serum albumin/PBST for 1 hr at room temperature. The filters were washed again as above and then preceded to chemiluminescence detection with a dioxetane-based substrate (Diagnostic Products Corporation, Los Angeles, CA, USA). The plaques exhibiting immunoreactivities were excised from the plates and the phages were converted into pBluescript phagemids (Fig. 7) by *in vivo* excision with Exassist™ helper phage, following the manufacturer's instructions. The excised phagemids were purified and subjected to automated DNA sequencing with M13 forward and reverse primers. The insert sequences were compared to the known sequences in the GenBank database with the BLASTN alignment algorithm<sup>31</sup>.

## **10. Sequence and 3D structure analysis**

The positive clones were subcloned to monoclonality, purified and excised *in vivo* to pBK-CMV plasmid forms (Stratagene). Plasmid DNA was prepared using a Quantum Prep Plasmid Mini- prep Kit (Bio-Rad, Hercules, CA). The

nucleotide sequence of cDNA inserts was determined by an ABI PRISM R310 Genetic Analyzer (PerkinElmer, Foster City, CA), and sequence alignments were performed with BLAST software and compared to sequences in the GenBank III. 3D modeling for allergenic proteins used PubMed, Entrez/Protein, Entrez/Nucleotide, PubChem, IBIS, VAST structure neighbors and the Conserved Domain Database<sup>14,15</sup>. 3D structures of biological units may be visualized using the 3D viewer Cn3D, which has recently been released as a new version v4.3, to support visualization of biological units with macromolecules generated via symmetry operations. Cn3D v4.3 also comes with a wider range of features, such as side-by-side stereo, and is distributed as a helper application for the web-browser. All 3D models and links to the NMR or X-ray crystal structures in the PDB are available from the SDAP website (<http://fermi.utmb.edu/SDAP/>). Our assessment of the quality of the 3D models using the available native structures was also compared with results from three assessment software tools, QMEAN<sup>32</sup> (<http://swissmodel.expasy.org/qmean/cgi/index.cgi>), ProSA<sup>33</sup> (<https://prosa.services.came.sbg.ac.at/prosa.php>) and Verify\_3D<sup>34</sup> (<http://nihserver.mbi.ucla.edu/SAVES/>) that provide quality scores based only on the 3D structures of the protein models.



*Nucleic Acids Research, 2012, Vol. 40, Database issue*

Figure 8. The colors used to depict 3d Domains in the sequence annotation graphic are independent of the colors used to depict molecules in the interactions schematic. The page provides links to the source database (PDB), taxonomy, PubMed, Entrez/Protein, Entrez/Nucleotide, PubChem, IBIS, VAST structure neighbors and the Conserved Domain Database. The interaction schematic shows that only the two largest protein subunits of the complex interact with the nucleic acids and that the smaller protein subunits surround the core of the complex and may interact with both or only one of the two large subunits.

### **III. RESULTS**

#### **1. Fine structure of salivary gland in the thorax of *Aedes togoi***

This study was undertaken to examine the morphology of collecting ducts and secretory cells of salivary glands using polyclonal antibodies generated in the mouse followed by confocal microscopy.

The paired salivary glands of mosquitoes are present in the thorax flanking the esophagus. Each gland has three lobes, two lateral lobes and one median lobe. In the female mosquito proximal, intermediate and distal regions form the lateral lobes. Each lobe has a central duct constituted by a layer of epithelial cells that are bound externally by a basal lamina (Fig. 9). The structure of the salivary glands probably influences the adaptation of their life cycle. The salivary gland consists of a pair of glands, each with two identical long lateral lobes and one short median lobe. All lobes are constituted by a single-layered epithelium of predominantly secretory cells (acinus) surrounding the secretory cavity and the salivary duct. The secretory cells from the distal part of female salivary glands (Fig. 9) synthesize the apyrase involved in blood feeding<sup>35</sup>. To identify the expression profiles of the saliva antigens in vivo, secretory cells and their surrounding tissues were immunostained. Adult female mosquitoes were used for evaluation of the salivary gland tissues in adult thoraxes. Saliva antigens were generally expressed in the surrounding cells near the endocytic vacuole (Fig. 9). Interestingly, expression of saliva antigen was not detected in the endocytic vacuole, but was only detected in the surrounding layer cells. Perhaps the surrounding layer cells are the cells that secrete saliva of mosquitoes were confirmed the fact. The endocytic vacuole layer was generally stained by phalloidine antibody used in this study. These in vivo results supported our findings on saliva antigen in vitro. Therefore it was suggested that anti-saliva antibody could be useful as a marker for mosquito saliva allergen (Fig. 10).

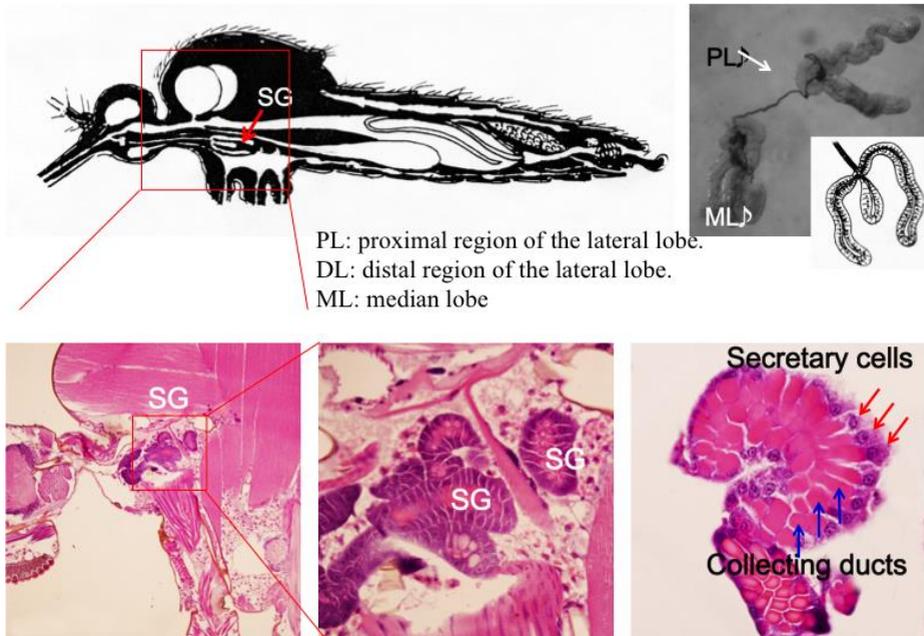


Figure 9. Immunohistochemical staining of mosquito salivary gland. The panel showed immunohistochemical staining samples. The specimens were cut in the longitudinal sections were stained with Hematoxylin and Eosin.

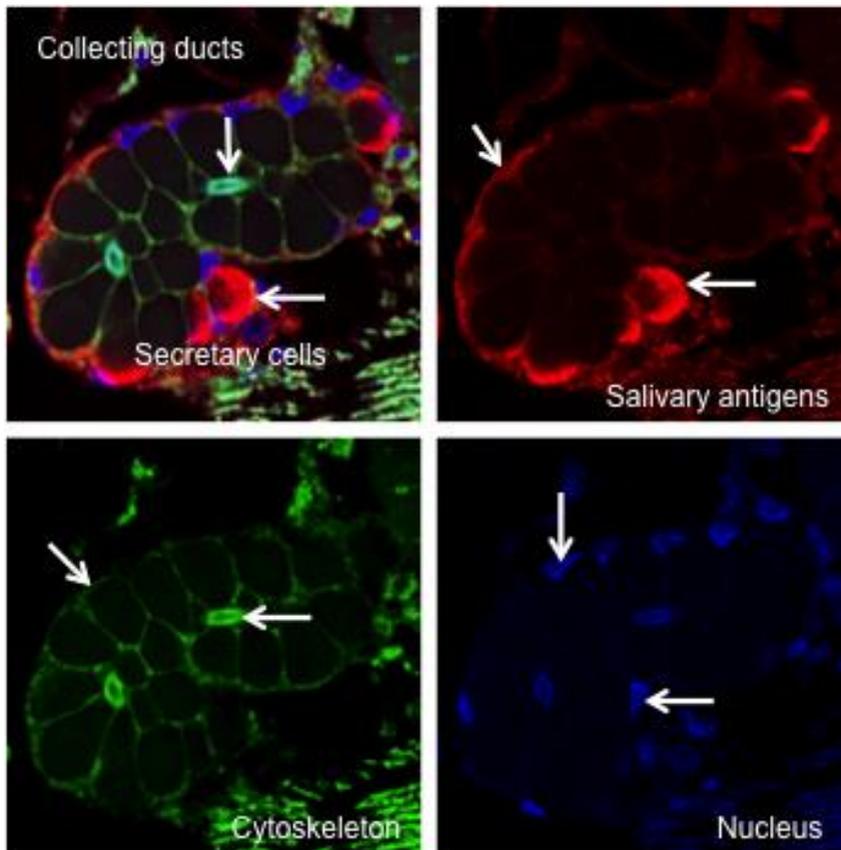


Figure 10. Confocal imaging of mosquito salivary gland. The tissue was treated with anti-SGE and anti-Phalloidin antibodies. SG proteins were detected as a red color using anti-mouse-Rhodamine (Red<sup>TM</sup>), Phalloidin was detected as a green color using anti-Phalloxin-fluorescein isothiocyanate. The cell nuclei were stained with DAPI. Samples were mounted for photography on a confocal microscope.

## **2. SDS-PAGE and Immunoblot Analyses**

The protein band patterns in whole body extracts and salivary gland extracts of *Aedes togoi* is illustrated in figure 11. Up to 19 polypeptide fractions were noted in the SGE and 26–30 polypeptide fractions were noted in the WBE. SGE was analyzed with the sensitized serum by immunoblotting. There were 9 reactive protein bands in the SGE of *Aedes togoi*. Specific mouse sera reacted to the proteins of 18.0, 33.0, 35.0, 37.0, 45.0, 57.5, 72.0, 90.0 and 150.0 kDa from *Aedes togoi*. The extract of *Dermatophagoides farinae* (DEF) was used as negative controls (Fig. 11). Immunoblot analysis disclosed that allergenic proteins in SGE of mosquitoes and their patterns were essentially mouse sera to the SGE of *A. togoi*.

## **3. Enzyme-linked immunosorbent assay**

For the determination of cross-reactivity the SGE of mosquito, antisera from the mice sensitized with *Aedes togoi* bites were used. The levels are shown in Figure 12. The serological activity against *Aedes togoi* was 1 µg/ml IgE concentration. Level check revealed a sufficient amount of IgE for immunoscreening. All of the control sera showed very low IgE levels.

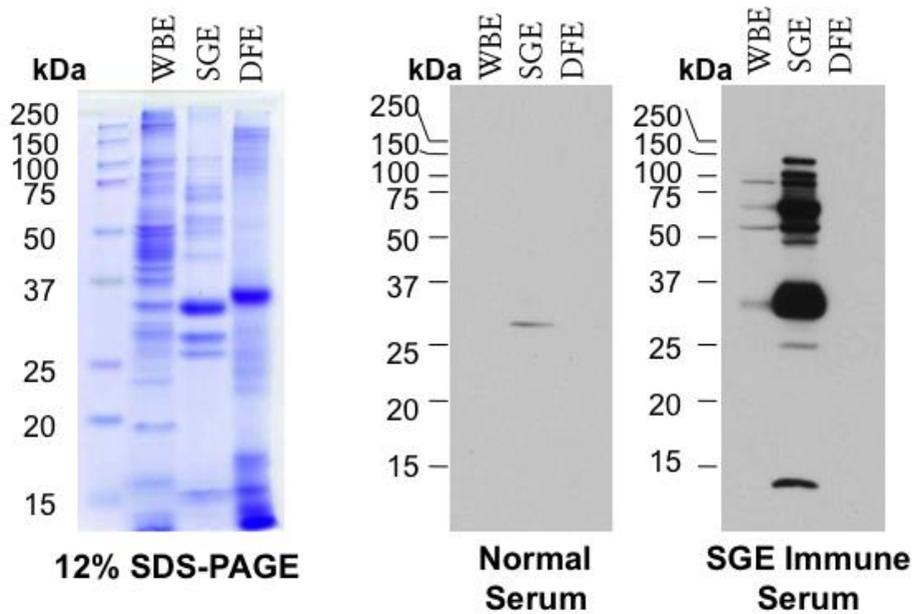


Figure 11. Protein electrophoretic profile of salivary glands of *Aedes togoi* and *Dermatophgoides farinae* & immunoblot analysis of SGE of the *Aedes togoi* using pooled mouse sera. Proteins were separated on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue and electrophoretically transferred to nitrocellulose membrane. Transferred nitrocellulose strips were incubated with pooled sera. Lane WBE, whole body extract of female mosquitoes at day 5 after emergence; Lane SGE, salivary glands extracts of female mosquitoes; Lane DEF, whole body extract of *Dermatophagoides farinae*; Lane left, Molecular weights markers of sizes (kDa) indicated on the left side of the picture.

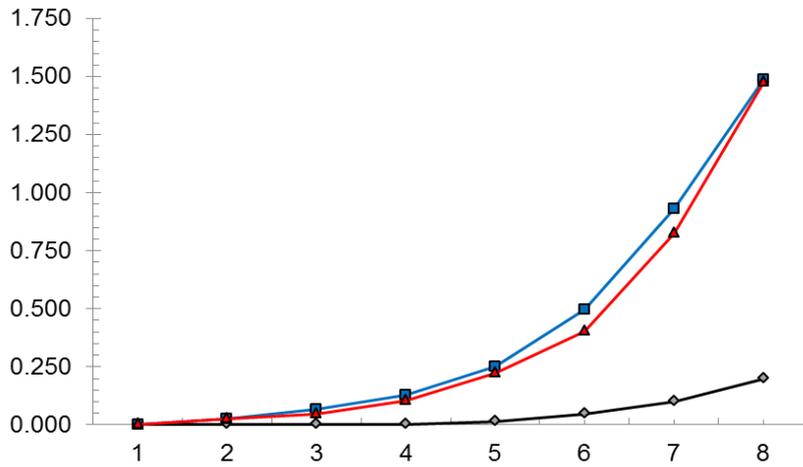


Figure 12. The mouse IgE levels in response to the SGE of *Aedes togoi* species by ELISA.

#### **4. RNA Extraction and construction of cDNA library**

High quality of RNA extraction is a primary requirement for various experiments in animal molecular biology such as gene cloning and characterization. *Aedes togoi* RNAs of high qualities (OD 260/280 = 1.81-1.93) yielding 0.56-0.78 mg/ml were isolated. Using 5 ug mRNA prepared, a primary and secondary cDNA strand was constructed to check whether they represents appropriate size of cDNA strands and cDNA was confirmed with agarose gel (Fig. 13). Then cDNA library was constructed. After plaque amplification, a cDNA expression library of  $1.2 \times 10^{10}$  pfu/ml and  $1.0 \times 10^9$  pfu/ml SM buffer were prepared from the mosquito salivary gland MSQ452 and MSQ549, respectively (Fig. 14). To check whether the library represents appropriate size of cDNA, some of clones were digested with *EcoR* I and *Xho* I. Most of clones are within the range of 0.2-0.8 kb, reflecting the abundance of cDNAs of this size in the library (Fig. 15).

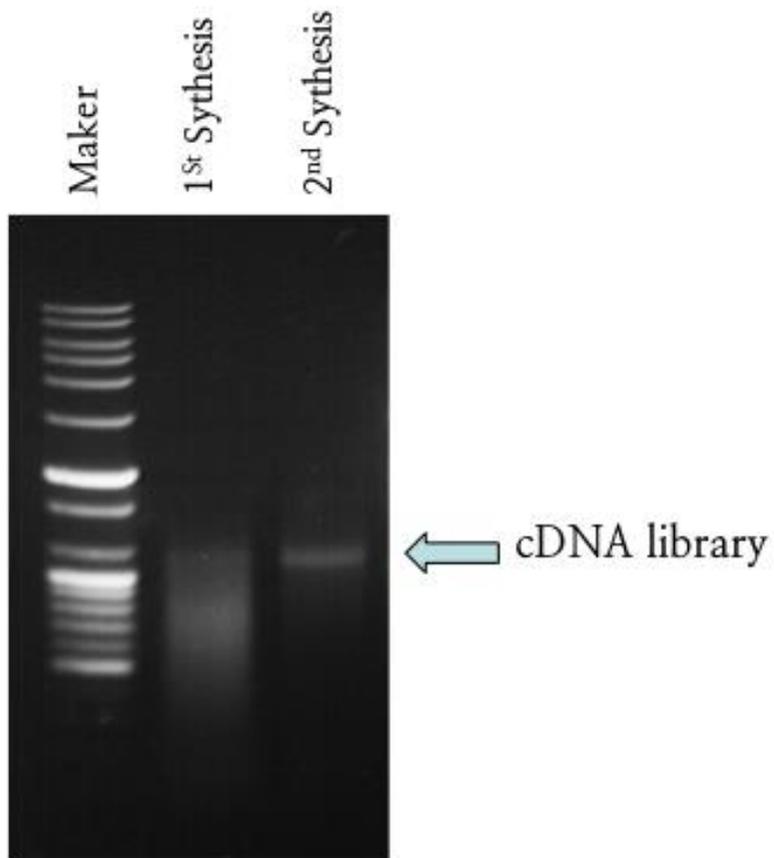


Figure 13. Quality check of the cDNA library from total RNA. Agarose gel shows the salivary gland cDNA samples from *Aedes togoi*.

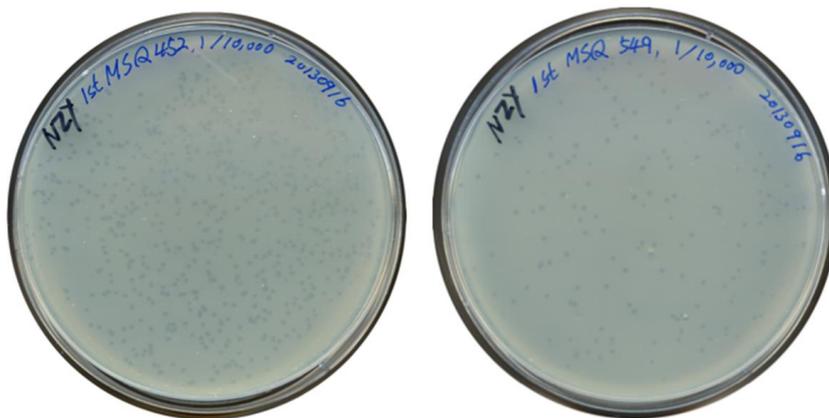


Figure 14. Amplified cDNA library. Lysis plaques of lambda phage on XL1-Blue MRF' *E. coli* bacteria.

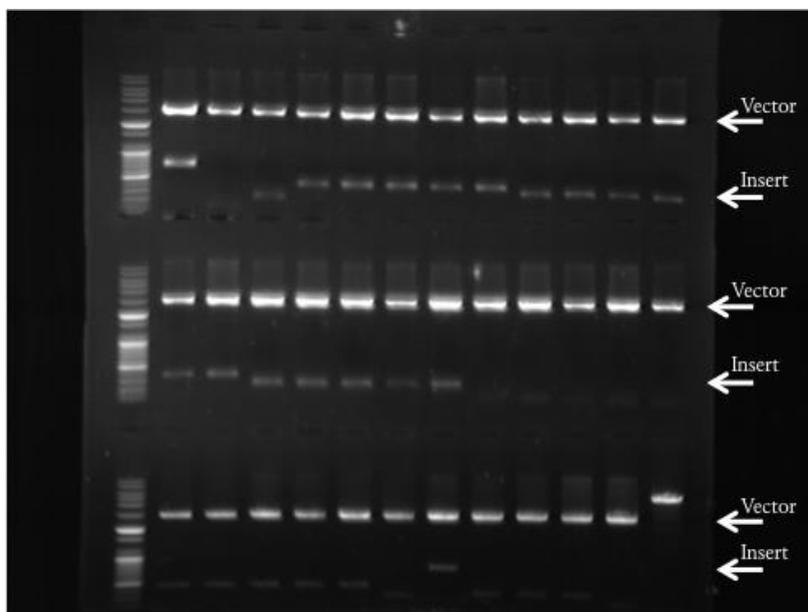


Figure 15. Agarose gel showing the restriction digestion of 6 cDNA with *EcoRI* and *XhoI* from *Aedes togoi* cDNA library. Left lane DNA marker.

## 5. Identification of saliva antigens by immunoscreening of mosquito salivary gland cDNA expression library with sensitized serum from *Aedes togoi*

A recombinant mosquito salivary gland cDNA library was screened with sensitized serum from *Aedes togoi*. In total, 4,000 clones were screened. Forty-five clones were found to have immunoreactivity. An example of a positive clone is shown in Figure 16. In order to confirm the identities of these positive clones, they were isolated and converted to phagemids. The inserts were then sequenced and compared to the GenBank database using the BLAST program. Four selected sequenced clones, which had more than 400bp of the insert, were analyzed. Four positive clones are known proteins (Table 2). Two of these proteins are protein of unknown function (#006 and #039), an Aspartate aminotransferase (AAT) superfamily of pyridoxal phosphate (#001), and Aldo/keto reductases, related to diketogulonate reductase (#015). Immunoscreening of  $4 \times 10^3$  clones with sensitized serum yielded a total of 45 positive clones (Fig. 16). As shown in Figure 17, the nucleotide sequences of the cDNA inserts identified 4 different genes, designated as #001, #006, #015 and #039.

Table 2. Proteins identified as saliva antigens by immunoscreening of the *Aedes togoi* cDNA expression library with sensitized serum.

Clone	Putative identification	Functional significance
#001	Aspartate aminotransferase	Enzyme in amino acid metabolism
#006	DUF2528	Protein of unknown function
#015	Aldo/keto reductases	NADPH-dependent oxidoreductases
#039	DUF1398	Protein of unknown function

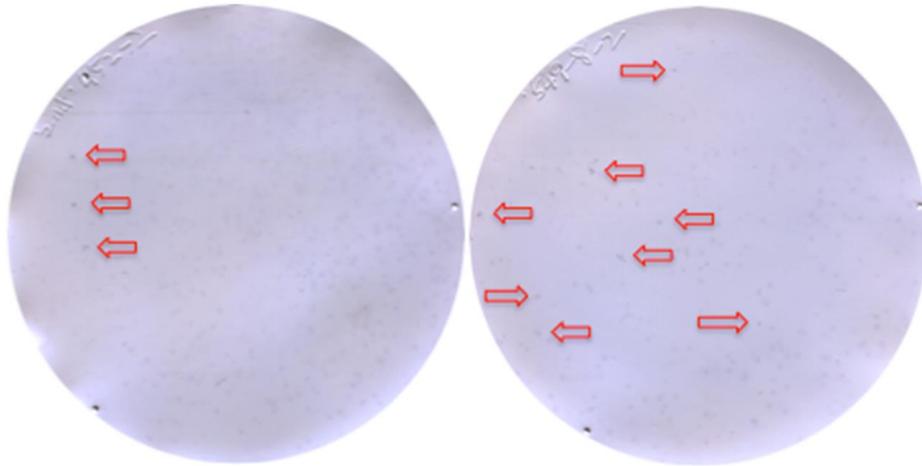


Figure 16. Demonstration of immunoreactive plaque. The arrow shows an immunoreactive plaque. An example showing a positive clone identified by immunoscreening of the *Aedes togoi* cDNA expression library. The black dot indicated by an arrow is the positive clone. Saliva protein-specific IgG can be detected in the sera of sensitized with mosquito bite. A  $\lambda$  phage cDNA expression library constructed from normal female mosquito was used to transduce *E. coli* for picoBlue immunoscreening. A representative primary screen used to identify immunoreactive phage plaques is shown.

## 6. Analysis of the sequence and 3D modeling

The profile of homology search results revealed that the nucleotide was of a deduced amino acid sequence of two proteins, DUF2528 and DUF1398 of unknown function, an aspartate aminotransferase, and Aldo/keto reductases cDNA in *Aedes togoi* (Fig. 17). The analysis of DUF2528 consists of dimer with alpha helices and beta sheets. Most non-local contacts involve the pairing interactions between beta strands and the packing interactions between helices

and beta sheets. The analysis of DUF1398 consists of monomer with alpha helices and beta sheets. Members of this family seem to be found exclusively in *Escherichia coli* and *Salmonella* species. The function of this family is unknown (Fig. 18 A and B).

The analysis of the sequence was performed using the NCBI/ BLAST network service of the National Center for Biotechnology Information. In many applications of 3D modeling for allergenic proteins, the goal is to determine the solvent accessibility of side chains on the allergens, and possibly predict the structure of conformational epitopes<sup>36</sup>.

(A)

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          10          20          30          40          50          60          70          80
gi 18978057 1 MSLYRKRRLRVKGEIYVWDSQGGKYYIDLIAGIGVNVILGHNHPKVVSELEQLEKLVVAGPMFDHEEKYEMLEELKFKVT 80
gi 14520659 1 MSLYRKRRLKIVRGEIYVWDSQGGKYYIDLIAGIGVNVILGHNHPKVVSELEQLEKLVVAGPMFDHEEKYEMLEELSRWVN 80
gi 14591479 1 MSLYRKRRLKIVRGEIYVWDSQGGKYYIDLIAGIGVNVILGHNHPKVVSELEQLEKLVVAGPMFDHEEKYEMLEELSKFVN 80
gi 57640210 1 MPLYRKRRLRVKGEIYVWDSQGGKYYIDLIAGIGVNVILGHNHPKVVSELEQLEKLVVAGPMFDHEEKYEMLEELSHWVD 80

          90          100         110         120         130         140         150         160
gi 18978057 81 YEYVYIGNSGTEAVEAALKFARLYTGRKEI I AMTNAFHGRTMGALSATWPKYKDFEPLVPGFKHIFPNVDEAAKEAIT 160
gi 14520659 81 FEYVYMGNSGTEAVEAALKFARLYTGRKEI I AMTNAFHGRTMGALSATWPKYKDFEPLVPGFKHIFPNVDEAAKEAIT 160
gi 14591479 81 FEYLYMGNSGTEAVEAALKFARLYTGRKEI I AMTNAFHGRTMGALSATWPKYKDFEPLVPGFKHIFPNVDEAAKEAIS 160
gi 57640210 81 YEYVYMGNSGTEAVEAALKFARLYTGRKEI I AMTNAFHGRTMGALSATWPKYKDFEPLVPGFKHIFPNVDEAAKEAIT 160

          170         180         190         200         210         220         230         240
gi 18978057 161 TETAAVIFPEIQEGGGVVPANEFFVKTLDLTDGKALLIADEVQSGLRGTGKFLAIEHYKVEPDIVTMGKIGNGVPPVSL 240
gi 14520659 161 RTTAAVIFPEIQEGSGIIPAKEEFVKTLDLTDGKALLIADEVQSGLRGTGKFLAIEHYKVEPDIVTMGKIGNGVPPVSL 240
gi 14591479 161 KETAAVIFPEIQEGSGVIPAKEFPMKALRDLTDGKALLIIVDEVQTLRGTGKFLAIEHYKVEPDIVTMGKIGNGVPPVSL 240
gi 57640210 161 KETAAVIFPEIQEGGGVVPANEFFVKTLDLTDGKALLIADEVQSGLRGTGKFLAIEHYKVEPDIVTMGKIGNGVPPVSL 240

          250         260         270         280         290         300         310         320
gi 18978057 241 TLENFDVERGKHGSTFGGNPLACKAVAVTLRLRREKLEIEKAAEKFIKGVENVVTRGKGLMIGLVKPKVAKVVEELQ 320
gi 14520659 241 TLENFDVERGKHGSTFGGNPLACKAVAVTLRLRREKLEIEKAAEKFIKGVENVVTRGKGLMIGLVKPKVAKVVEELQ 320
gi 14591479 241 TLENFDVERGKHGSTFGGNPLACKAVAVTLRLRREKLEIEKAAEKFIKGVENVVTRGKGLMIGLVKPKVAKVVEELQ 320
gi 57640210 241 TLTDLFPRGKHGSTFGGNPLACKAVAVTLRLRDRDLVEKAGEKFMFSGERVVTRGKGLMIGLVLRPAGNYVKALQ 320

          330         340         350         360
gi 18978057 321 NRGYLVHTAGQRVIRLLPPLIIISKDEINQAKSAIEGVINDIYGR 364
gi 14520659 321 NRGYLVHTSGQRVIRLLPPLIIISKEMMEVKSALIEGVINDGTEG 364
gi 14591479 321 NRGYLVHTAGQVRMLRLLPPLIIISKETMQDVKLALIEGVINDLRGG 364
gi 57640210 321 ERGILVNTAGNRVIRLLPPLIIIEGDTLEEARKEIEGVINDIIL-- 362

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(B)

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gi 75090053 14 KASIEIEIDHDVM.[1].EEKLHQINNFWSDSE.[3].NKHGS.[1].LNAVLIMLAQHALLIAISS.[6].VVCFEDWN 84
gi 75081097 35 SYEIVVDVHDHNL.[1].EEKLCELVRWFSEGD.[3].ERHGP LQAFKLFARFLAATVEE ISPQDAFN 98
gi 134288799 14 SYEIVVDVHDHIL.[1].EEKLCELVRWFSEGD.[3].ERHGP LQAFKLFARFFAATVEE LSPKDAFN 77
gi 87119988 4 AFKMFVKGIDEQKL NEILPELFSFVGWSE EDDKD.[1].LYKLLQTLSCVFSMGIQG KSLESVLR 64

gi 75090053 85 .[1].GNGQEGWPSMDGSEGRITDIDTS.[1].IFDSDDMTIKAA 122
gi 75081097 99 AGRIEGPPVVDGSSGLRVVVEDEF SFEADDIVLIEI 134
gi 134288799 78 AGRIEGPPVVDGSSGLRVVVDDEL SFEADDIVLIEI 113
gi 87119988 65 .[2].GVGEGWPQMDGSGWIEITSIEAF MSDTDDPHVEAV 102

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(C)

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gi 586553 20 .[90].DRSLNESLKALGLEVDLLQHPW.[35].ETYKQLEKIYLDP.[3].RVRAIGVSNFNSIE.[138].335
gi 16263625 15 .[99].ERQLRQSRERLRWRERIDVLQVHSL.[4].QVRHWLAHKKAEG SRYRIGITQWSP.[154].321
gi 16265111 14 .[93].AAQIEASKDYGVKRFKDLMLQVHNL.[4].EHLPALFDKKAAG RLYRVGITTSRGR.[129].289
gi 15899596 1 .[92].LKASEGLKRLNTSYIDLYLVHWP.[7].ETAKAMERLFNEG KIRYVGLSNFSLP.[155].304
gi 13541706 1 MKAREASLNKLGIDYLDLYQFNWP.[7].ETMKATEELMDEG KIKNIGINNFSK 57
gi 13541983 7 .[97].IKACKASLSRLNTKYVDLYQHFP.[7].ETFKAMEKLVDDG LVKFIGVSNFSRG.[128].288
gi 16081208 3 .[86].LKSCEESLKKLGTDVVDLYQLHWP.[7].ETMSAMEKLVDEG RIRYIGVSNFNSVK.[139].284
gi 16081541 12 .[97].IKACKRSLKRLNMKYIDLYQHFP.[7].GTLRAMEKLVDDG LRYRIGVSNFNLD.[130].295
gi 15789702 3 .[88].APATEQSLKELGVDYVDLYLVHWP.[7].ETLAALADLQADG VTERIGVSNFPR.[129].276
gi 15790128 300 .[88].REACTGSLDELVAADFDCYALHWP.[41].ETWRMEALHDDG LARSLVGCVNTP.[122].600

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(D)

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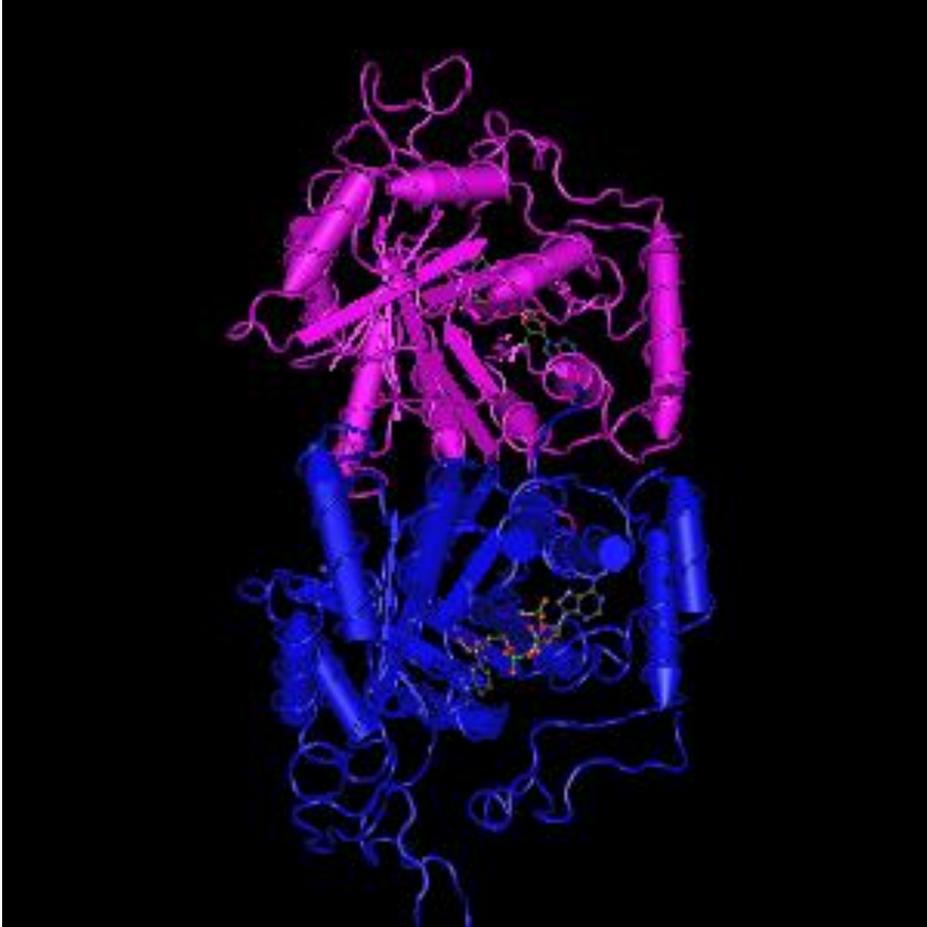
          10          20          30          40          50          60          70          80
2HH8_A 8 QVVFQKIFDKVRNDLNYQWFYSELKRNHNSHYIYLLATENVHIVLKNMNTVLLKGLKNIVSVKFSKDRHLIETSNK 87
gi 160331909 3 QVAIFKEIFDQVRKDLNCELFSYSELKRNHNSHYIYLLATDNIHIVLENDNTVLLKGLKVVNVKFSRMTHLIETS 82
gi 75482843 3 LQMLNHHFFERVRKDNANFLIDLLENNYIAIYFVATGNVKITHTGHFISIKSNRKLKVNSTPMTQLIKITSAKHF 82
gi 152970056 3 LLVRLGHFFEQARSADAYATFANELRRHQVSYIYFVSTGNMNFVMADEVVSVKARGLLRVRAEASHCLTRAAVIRHF 82
gi 91210516 8 QIAIFKQIFDKVRNDLNYHWFYSELKRNHNSHYIYLLATENIHLVLENDNTVLLKGGQGVNVNRSKKNKLEATL 87
gi 293409532 4 QVVVFQKMFQVRKEQNFQWFSYSELKRNHNSHYIYLLATDNIIRITHDDTVLLRGTNRLLKVTSTKPKAKIEALLHI 83

          90          100         110         120
2HH8_A 88 SREITFQYRRNLAKAGVFRVNTIHEQKRYYYTFDNSLLFTESI 132
gi 160331909 83 SREITFQYRNLAKAGVFRVNTIHEKRYYYTFDNSLLFTESI 127
gi 75482843 83 SGEHSYEKYCTDLATAGVFKWIVLGNQTRQVWSKDNQLLYIENV 127
gi 152970056 83 ARAINFEQYCRDLASAGVFKWIVDLEETRHYWSKDNLLYKECL 127
gi 91210516 88 SGEHSFYERKNLATAGVFRVNTIHEKRYYYTFDNSLLFTENI 132
gi 293409532 84 CGKSTFPEYCSYLAGAGVFRVNTVNNHNRKSYAIDNTLLYIEDV 128

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Figure 17. Alignment of deduced amino acid sequences of the partial # 001, # 006, # 015 and # 039 saliva antigen genes of the *Aedes togoi*. (A) Amino acid sequence comparison of the # 001 immunoreactive plaque; aspartate aminotransferase (AAT) superfamily (fold type I) of pyridoxal phosphate (PLP), (B) The # 006 immunoreactive plaque; protein of unknown function (DUF2528), (C) The # 015 immunoreactive plaque; aldo/keto reductases, related to diketogulonate reductase, (D) The # 006 immunoreactive plaque; protein of unknown function (DUF1398).

(A)



(B)



Figure 18. 3D structure of the protein of unknown function. (A) #006\_Protein of unknown function (DUF2528), crystal structure of *saccharomyces cerevisiae* arabinose dehydrogenase Ara1 complexed with NADPH. (B) #039\_Protein of unknown function (DUF1398). This family consists of several hypothetical Enterobacterial proteins of around 130 residues in length.

#### IV. DISCUSSION

Mosquitoes are very important from the standpoint of human health because female mosquitoes suck blood from animals including human, and they serve as vectors in the transmission of several important human diseases. Mosquito larvae occur in a variety of aquatic habitats, such as ponds, pools of various sorts, artificial containers, tree holes, streams, rice field, etc., and each species breeds only in a particular type of habitat. In Korea, 9 genera and 48 species of mosquitoes have been described<sup>37</sup>. The *Aedes togoi* species is distributed on most islands and at the seaside. The *Aedes togoi* breeds only in the rock pool of seaside (Fig. 3). However, the allergenicity of salivary gland extracts of Korean mosquitoes in humans and experimental mice has not been reported until now. This species is thought to lay 30-80 eggs and overwinters in the egg stage, however, during a warm winter they may also overwinter as larvae<sup>38</sup>. The allergic problems of *Aedes togoi* may occur mostly in coastal areas.

In the present study, many proteins in the *Aedes togoi* were detected in the whole body extract and salivary gland extract, and some 20 proteins were shown in the WBE and SGE by Coomassie brilliant blue staining (Fig. 11). These results are somewhat different from previous studies, which have described high molecular weight protein fractions of the *Culex pipiens* species. These differences in the high molecular weight proteins between the *Culex pipiens* species and *Culex pipiens pallens* may reflect a different subspecies of mosquito. However, the low molecular weights of the proteins with 18 and 33 kDa were similar to those in the previous study<sup>39</sup> (Fig. 11).

Mosquitoes take blood meal for laying egg and their development. In this study, the salivary glands consist of a pair of glands, each with two lateral lobes and one median lobe in *Aedes togoi* (Fig. 9). The proximal part contains enzymes such as  $\alpha$ -glucosidase,  $\alpha$ -amylase and maltase involved in the metabolism of sugar meals, which are also found in the secretory cells of male

salivary glands. The median lobe, in addition to secretory cells, contains nonsecretory cells implicated in fluid transport<sup>40,41</sup>. The adult female appears to have some control over the contents of released saliva (activities of maltase or apyrase enzymes), depending on whether she is feeding on sugar or blood<sup>42</sup>. The salivary glands, like other organs in the hemocele, are covered by a basal lamina that is external to the basal surface of the glandular cells. The apical surface of the secretory cells is indented by a large secretory cavity that is a part of a continuous cavity indenting all cells<sup>40</sup>. The secretory duct where the saliva comes from and the path from the secretory cavity to the proboscis differs between *Aedes* and *Anopheles*. In *Aedes*, the chitinized duct continues to the end of the distal portion of the gland<sup>40</sup>; in *Anopheles*, it stops in the intermediate zone<sup>40</sup>. It is unknown if the end of the duct is open or closed. The duct wall in *A. aegypti* is thinner in the distal than the proximal region of the lateral lobes<sup>40</sup>.

The skin reactions to mosquito bites are derived from salivary secretions and consist of an immediate and a delayed reaction with the involvement of IgE mediated and lymphocyte-mediated hypersensitivities<sup>11,43</sup>. In the saliva of *Aedes aegypti*, a 68 kDa protein was identified as apyrase, an ATP diphosphohydrolase. The role of this enzyme in mosquitoes seems to be related to the anti-platelet aggregating activity found in their salivary secretion or salivary gland homogenates<sup>42</sup>.

It has been demonstrated on protein patterns of mosquitoes that there are more than 20 protein fractions in the saliva of *Aedes togoi* using silver staining<sup>44</sup> and 14- to 68-kDa protein fractions in the saliva of 4 *Aedes* and 3 *Culex* spp. using colloidal gold staining<sup>39</sup>.

In the present study, BALB/c mice bitten by the mosquito had significantly higher anti-mosquito IgE antibody levels than those mice not bitten by the mosquitoes (Fig. 12). These findings suggest that IgE-mediated hypersensitivity is involved in the development of mosquito allergy. The results of mosquito-specific IgE levels in mice show that five sera had positive titers

against only mosquito antigen when the serum was detected 0.625ng/ml. It is suggested that a range of dose used for a species-specific IgE test is useful in 0.625ng/ml (Fig. 12).

Several investigations based mainly on Western blot analysis data have described specific IgE-reactive proteins in mosquito saliva and salivary gland extracts. *Aedes togoi* mosquito-sensitive individuals revealed IgE-binding bands with apparent molecular weights of 18.5, 30.5, 33, 37 and 57.5 kDa<sup>13</sup>.

In the present study, data obtained from Western blot with the pooled serum of the sensitized to *Aedes togoi* were in agreement with the IgE-reactive protein fractions from 18.0 to 57.5 kDa, but there was no reaction with 72.0, 90.0, and 150.0 kDa. In particular, the 18.0, 33.0, 37.0 and 57.0 kDa fractions elicited strong IgE responses (Fig. 11). This suggests that these four protein fractions may be major IgE-binding proteins in salivary gland extracts of *Aedes togoi*<sup>13</sup>.

In our study, which was performed according to a method of Harlow and Lane<sup>45</sup>, there were no bands in the negative sera (Fig. 11). Harlow and Lane stated that if background bands are generated due to a secondary reagent, exchange of an alternative label or adsorption of the secondary reagent with liver acetone powder is recommended. Liver acetone powder was used to reduce background bands in this experiment.

In the mouse, interleukin-4 preferentially induces isotype switching to IgG1 and IgE<sup>46</sup>. A previous study showed that human IgE and IgG4 antibodies of mosquito bite-sensitive children bind to the same saliva proteins as antibodies from the immune animals<sup>39</sup>.

As shown in this study, the sensitized mice showed IgG1 antibody responses to major salivary gland extracts of *Aedes togoi*, similar to IgE (Fig. 11, 12). Thus, anti-mouse IgG1 reactivity to the salivary gland extracts of mosquitoes could be useful to identify allergens by Western blot analysis.

Cross-reactivity with respect to bite reactions caused by different mosquito species has been reported previously<sup>47</sup>. In the present study, an ELISA inhibition

experiment with sera immune to *Aedes togoi* revealed that the mouse IgE level decreases dose dependently with SGE of *Aedes togoi* (data not shown), whereas it does not decrease in *Dermatophagoides farinae*. It is suggested that species-shared allergens may not exist within these mosquitoes and house dust mite.

The usual approach to isolate a recombinant DNA clone encoding a specific allergen sequence is to screen a recombinant cDNA library. Serological screening of recombinant cDNA expression libraries has been widely used for the identification of allergens in various allergy types. Identification of allergens in mosquito may facilitate the development of immunotherapies and biomarkers. A recombinant cDNA library consists of a large number of mosquito salivary gland, each one of which contains a different segment of saliva allergen DNA.

The purpose of our investigation is to identify allergens in mosquito salivary glands by using the serological analysis of recombinant cDNA expression libraries method. A recombinant mosquito salivary glands cDNA expression library was screened with pooled sera from five sensitized mice (Fig. 13-16).

The examination of 3D structure often provides explanations for patterns of sequence conservation observed in protein families, and 3D structure alignment can serve as a guide for constructing accurate multiple sequence alignments such as used in phylogenetic analysis. Recently the PDB has begun to provide comprehensive data regarding the biologically relevant assemblies/ complexes or quaternary structures of the macromolecules described in its records<sup>48</sup>.

Four IgE response antigens encoded by two known and two unknown genes were isolated, including aspartate aminotransferase (AAT) superfamily (fold type I) of pyridoxal phosphate (PLP) and Aldo/keto reductases, related to diketogulonate reductase. AAT is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells, and it is commonly measured clinically as a marker for liver health.

A domain of unknown function (DUF) is a protein domain that has no

characterized function. A number has collected these families together in the Pfam database using the prefix DUF followed, with examples being DUF2528 and DUF1398 (Fig. 17, 18). Accordingly, the NCBI Molecular Modeling Database<sup>49</sup> has been enhanced to emphasize the functional molecular complex (i.e. quaternary structure) and the interactions between its molecular components (Fig. 8).

## V. CONCLUSION

The salivary glands of adult *Aedes togoi* are paired organs, located in the thorax and composed of two identical lateral lobes and a shorter and wider median lobe. Protein band patterns of the WBE and SGE were different from one another. We confirmed the morphology of collecting ducts and secretary cells of salivary glands. Specific mouse IgE reacted to the protein in SGE of 18.0, 33.0, 35.0, 37.0, 45.0, 57.5, 72.0, 90.0 and 150.0 kDa from *Aedes togoi*. Two protein of unknown function was detected from this study. In summary, we demonstrated that many candidate saliva antigens could be identified in the cDNA library screening of *Aedes togoi*. Our findings warrant further investigations on these proteins, aiming to elucidate their immunogenicity in allergens. Analysis of the 3D structure of DUF1398 and DUF2528 was not similar with any other allergens identified in plants or animals, despite low sequence identities to their templates, the global folds of the 3D models of the cockroach allergen *Bla g 4* and the mosquito salivary protein antigen *Aed a 2* had a sizable fraction of structural overlap, suggesting that it would be a potential target for therapeutic agents in specific mosquitoes bite allergens.

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## ABSTRACT (IN KOREAN)

진단 및 면역치료 시약 개발을 위한 모기 침샘 내 항원에  
대한 생쥐의 체액성 면역 반응

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최 경 민

모기의 주둥이는 자흡형으로서 흡혈시에 숙주의 피부속으로 주둥이를 삽입하고 혈액응고 방지를 위하여 항 응고제가 포함된 타액을 주입한다. 이 경우 대부분의 사람에서 과민면역반응을 나타내어 소양감, 홍반, 구진, 궤양성구진, 및 수포형성등 다양한 국소 피부반응과 심한경우 전신반응을 일으킨다.

본 연구는 국내서식하는 토고숲모기(*Aedes togoi*)를 대상으로하여 타액선을 분리하고, 유전자 서고를 확립한 후, 모기에 노출된 실험 쥐로부터 그 혈청을 확보하고, 타액 알레르기 원인 항원의 유전자 특성을 규명하였으며, 재조합 특이 모기타액 단백질을 제조할 수 있는 기반 기술과 유전자를 획득하고자 하였다.

그 결과 토고숲모기 타액 항원은 18.0 kDa, 33.0 kDa, 35.0 kDa, 37.0 kDa, 45.0 kDa, 57.5 kDa, 72.0 kDa, 90.0 kDa 및 150.0 kDa의 분획이 모기 알레르겐의 주요 항원으로 추정되었다. 확보된

모기 침샘 유전자 서고와 모기 타액에 감작된 실험쥐 혈청을 이용한 면역스크린법 으로부터 양성반응을 보인 45개 플라크중에서 2개의 알려지지 않은 단백질 코드 유전자를 확보하였다. 새로이 확인된 2개 DUF1398 와 DUF2528의 단백질을 3차원적 구조를 분석한 결과 식물성 알레르겐이나 동물성 알레르겐과 일치된 결과를 얻지 못하였으나, 3차원 모델의 구형 접힘구조에 있어서 그 유전자의 염기서열의 유사성과는 상관없이 이전에 알려진 바 있는 바퀴 알레르겐 *Bla g 4* 및 모기 침샘 알레르겐 *Aed a 2*와 부분적으로 그 구조가 일치함을 확인하였다. 이는 잠정적으로 알레르겐의 후보 물질로 간주되며, 지속적으로 연구해야할 표적 물질로 판단된다.

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핵심되는 말 : 알레르겐, 모기, 토고숲모기, 침샘, 면역글로브린 E