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Immunogenic Protein from Salivary Gland of *Aedes aegypti* Against to Human Sera

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ABSTRACT

Dengue Haemorraghic Fever (DHF) is an acute Flavivirus infection transmitted by several species of Aedes mosquitoes with Aedes aegypti as the main vector. During blood feeding, arthropod vectors inject saliva into vertebrate hosts. The saliva is biochemically complex, pharmacologically active and plays an important role in pathogen transmission. The objective of this research is to identify immunogenic salivary gland proteins of Ae. aegypti against human blood sera of people living in endemic area. Protein profile from Salivary Glands (SG) of Ae. aegypti was observed by 12% SDS-PAGE from lab. scale cultures and from landing populations. Identification of immunogenic proteins from both sample was carried out by using Western Blot Analysis after cross reaction of Salivary Gland Extract (SGE) with 3 different human sera: from DHF patients, healthy persons who were exposed to Ae. aegypti and healthy person who were likely not exposed. Sera from healthy people from non endemic areas and from infants were used as negative controls. Over all, the protein profiles from lab. scale cultures SGE and landing populations were quite similar. We found 13 protein bands were identified ranging from 26 kDa up to 255 kDa. We predicted that 255, 56, 31, 27 and 26 kDa are target protein, which is two of immunogenic proteins were able to cross-react with human sera from people living in endemic area on 31 and 56 kDa. These bands appeared only in samples from humans who were previously exposed to mosquitoes bites, and not in humans who had not been exposed. These immunogenic salivary proteins may serve as human immune response against Ae. aegypti bites. This result indicated that may the 31 and 56kDa protein has function as transmitted pathogens.

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INTRODUCTION

Dengue Fever (DF), especially the more severe manifestation Dengue Haemorrhaghic Fever (DHF) and Dengue Shock Syndrome (DHF/DSS) are caused by Dengue Virus (DENV). There are four serotypes of the virus and these are referred to as DENV-1, DENV-2, DENV-3 and DENV-4 (Edelman 2007; Whitehead *et al*. 2007; Murell *et al*. 2011). Dengue fever is disease an important public health problem in tropical climates and appears to be spreading to new areas. These infections have been reported in over 100 countries in tropical and subtropical regions, such as Southeast Asia, Pacific island, central and south America and Africa (Smith and Deen 2008). About 50-100 million people are infected by Dengue Fever and approx. 500.000 cases are fatal. All together 2.5 billion people live in areas where dengue is endemic DHF have become increasing mortality and cause of morbidity, thus are responsible for major economic losses mainly in developing countries (Edelman 2007; Abreu and Ortigao 2010).

DENV is transmitted by several species of *Aedes* mosquitoes with *Aedes aegypti* as primary vector and *Aedes albopictus* as a secondary vector (Sim and Dimopoulos 2010). Mosquitoes generally acquire virus while blood feeding of a person infected with dengue virus and transmits it again to a healthy persons (Almeras *et al*. 2010). Blood feeding required for nutrition, egg development and survival (Andrade *et al*. 2005). Vertebrate hosts have three systems that play a role in inhibiting the blood feeding process, these are haemostasis, inflammation and immunity. Haemostasis is host response related to control blood loss due to vector bites which includes following mechanisms i.e. thrombocyte aggregation, blood coagulation and vasoconstriction. Inflammation is a host response caused by tissue damage, characterized by pain, skin rashes and heat, due to vasodilatation. Immunity related to host immune response against exposure of antigens in the interaction between vector and host (Ribeiro and Francischetti 2003).

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To counter-attack the host mechanism for inhibiting blood feeding, SG of arthropod vectors has developed molecular components i.e. vasodilators and immunomodulators. Vasodilator factors potentially inhibit homeostasis. They function as an anti-coagulants and inhibit vasoconstriction. Immunomodulatory factors can induce the host immune response which will be manifested as an allergic response in the skin that is itchiness and redness (Andrade *et al*. 2005; Tangamani and Wikel 2009; Fontaine *et al*. 2011). Some research showed that the saliva of mosquitoes contains also immunogenic components (Gillespie *et al*. 2000; Remoue *et al*. 2006, 2007), this has been proven by the development of a natural antibody response in people living in endemic area which is due to frequent exposure to salivary components (Cornelie *et al*. 2007). This indicates that the vector bites may have a positive effect on the host immune response. Other results showed that exposure to uninfected *Phlebotomus* caused resistance to *Leishmania major* due to an increase in cytokines that related to cellular immunity (Kamhawi 2000). Another report on murine models showed that mice exposed to *Ae. aegypti* saliva, gave the same effect by modulation of cytokine systemic response (Schneider *et al*. 2004). In the case of malaria, Donovan *et al*. 2007, reported that animal models previously exposed to *Anopheles stephensi* can enhance their immune response and thus inhibit the development of the parasite in their liver and blood after challenge by malaria parasites. Further evidence showed that haematophagous arthropod saliva contains active protein components that are able to modify the haemostasis and cellular immune responses and thus induce specific IgG antibodies in people living in endemic areas. (Fontaine *et al*. 2011).

Since the potential and important role of salivary gland proteins to increase pathogen transmission into the host, many researchers have focused to identify and characterize molecules in the salivary gland of arthropod vectors (Andrade *et al*. 2005). Transcriptomics and proteomics analysis by mass spectrometry have successfully identified 24 proteins such as. apyrase, serpin 1 and 2, D7 protein, adenosine deaminase (ADA), a serine protease, amylase, actin, purine nucleosidase, lectin (Ribeiro *et al*. 2007). Almeras *et al.* 2010 have identified by the same approach, 120 SG proteins from *Ae. aegypti*. 15 proteins were identified as secretory proteins involved in the process of blood feeding. Some secretory proteins have been described as a proteins that can modulate the immune response, these are D7 protein (37kDa), adenosine deaminase (ADA), purine hydrosilase, apyrase (68kDa), α-glucosidase (67 kDa), 30 kDa allergen (Peng and Simons 2004). Other proteins in SG of *Ae. aegypti* has an anti-haemostatic activity i.e. Aegyptin, which is a secretory protein family members of the 30-kDa allergen. This protein consists of the important amino acid such as glycine, glutamic acid and aspartic acid. Aegyptin is facilitating the process of blood feeding indirectly, enhancing pathogen transmission from vector to host and was also proven as an allergen (Calvo *et al*. 2007). SG, specifically the immunomodulatory factor, is the determining factor increasing the transmission of the pathogen to the human host. However, specific components in SG that mediating this process has not been identified so far. The study of immunogenic protein in the salivary gland of *Ae. aegypti* will be the initial step to identify immunomodulatory factor that potentiate pathogen transmission. Especially considering that the study of immunogenic proteins against human blood sera in Indonesia, as an endemic area has never been conducted. Therefore, this study is an important step to determine the potential use of SG from *Ae. aegypti* as target in developing vector-based vaccine to inhibit pathogen transmission to humans.

MATERIALS AND METHODS

1. Rearing of Ae. aegypti (lab scale cultures & landing populations) and Salivary Gland (SG) Dissection:

Mosquitoes larvae were collected and reared under strictly identical standard conditions at 28°C and 60% relative humidity at Zoology Laboratory of Biology Department- Faculty of Mathamatic & Natural Sciences, Jember University. Mosquitoes were supplied with a cotton wool pad soaked in 10% sucrose solution. Female *Ae. aegypti* mosquitoes were selected 7–10 days-old after their first blood feeding on rabbit blood maintained at 37^{o}C (Almeras, *et al*., 2010). The salivary glands from adult mosquito females were dissected using a fine entomological needle under a stereomicroscope at 8x magnification. The isolated salivary glands were pooled into a microcentrifuge tube on ice in Phosphate-Buffered Saline (PBS) and PMSF, then stored frozen at -20^{o}C until needed.

2. Salivary Gland Protein Extraction(Lormeau 2009):

Salivary glands in PMSF and PBS were added with lysis buffer (1:1) containing 1.5 mM MgCl2, 10 mM tris HCl, 10 mM NaCl, 1% Nonidet P-40, 2 mM EDTA NaOH. Mixture were homogenized by using micropistile and then sonicated in the water bath for 30'. Supernatant was collected after centrifugation at 12.600 rpm for 15', 4 ^{o}C **(**Lormeau 2009). Protein extract was concentrated by using eppi-membran and centrifugated at 10.000 rpm, 4 ° C. For our analysis, we use protein extract from salivary gland with the concentration of 0.69 µg/µl. Salivary gland proteins were then stored at-20^{o}C until used.

3. Blood Sera Collection:

Sera sample were taken from endemic area around Jember, East Java i.e. DHF patient, healthy person who were exposed by *Ae. aegypti* and healthy person who were likely not exposed by *Ae. aegypti* bites were collected from adult aged 15-40 y.o. Sera from healthy adult residents living in non-endemic region (sub tropical area) and sera from infant were used as negative control. All participants gave their informed consent to take part in the study. The collecting protocol was approved by the Ethical Committee of Medical Faculty, University of Brawijaya -Malang, Indonesia.

4. SDS-PAGE & Western Blotting:

Total protein from salivary gland extract were analysed by 12% SDS-PAGE by staining the gels with Commassie brilliant blue (CBB) R-25 to visualize the proteins. Proteins were transferred to a PVDF membrane under constant current (100 MA) for 1 hour by using semidry Western Blotting. The membranes were blocked at room temperature for 1 hour with 5% non-fat dry powdered milk in 1x TBS. After washing thoroughly, the PVDF membrane was treated with the pooled sera at the dilution of 1:500 and incubated overnight at 4^{o} C. PVDF membranes were then incubated with secondary antibodies anti-human IgG antibodies (goat) AP-conjugated (1:5000) for 2 hours. Color development was done with NBT-BCIP Phosphatase substrate. Prestained broad range molecular weight markers (7-250 kDa) (Intron cat 24084, 24085) were used for estimating protein sizes.

RESULTS AND DISCUSSION

The salivary glands of adult female *Ae. aegypti* have a distinctive tri-lobed structure consisting of a single medial and two lateral lobes, lateral lobe defined in two regions Proximal Lateral (PL) and Distal Lateral (DL) (Fig. 1). *Ae. aegypti* salivary gland is pairs in structure, and connected by salivary duct (Juhn el al. 2011). This salivary glands produce proteins which contain a number of pharmacologically active components that counteract vertebrate hemostasis, thus allowing the mosquito to feed successfully. These proteins act as vasodilator, anticlothing, antihemostatic protein and play a role in pathogen transmission as well as to induce immune response in the vertebrate host (Valenzuela *et al*. 2002, Waitayakul *et al*. 2006; Cornelie *et al*. 2007)

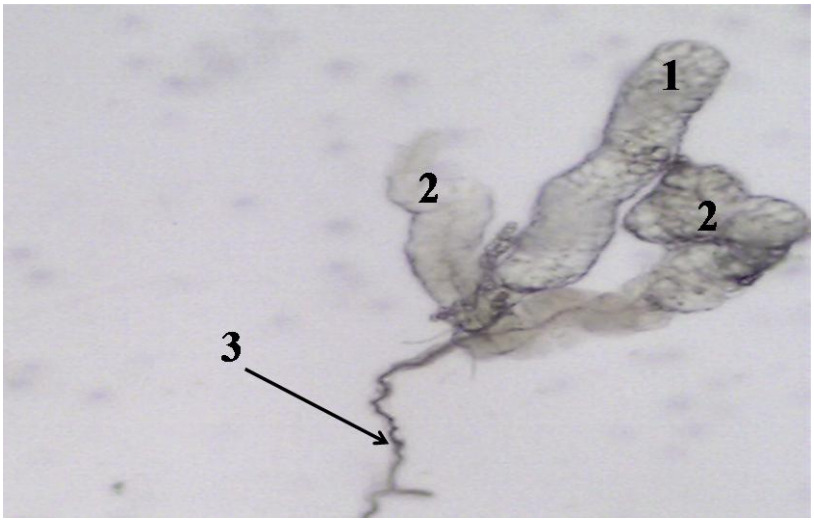
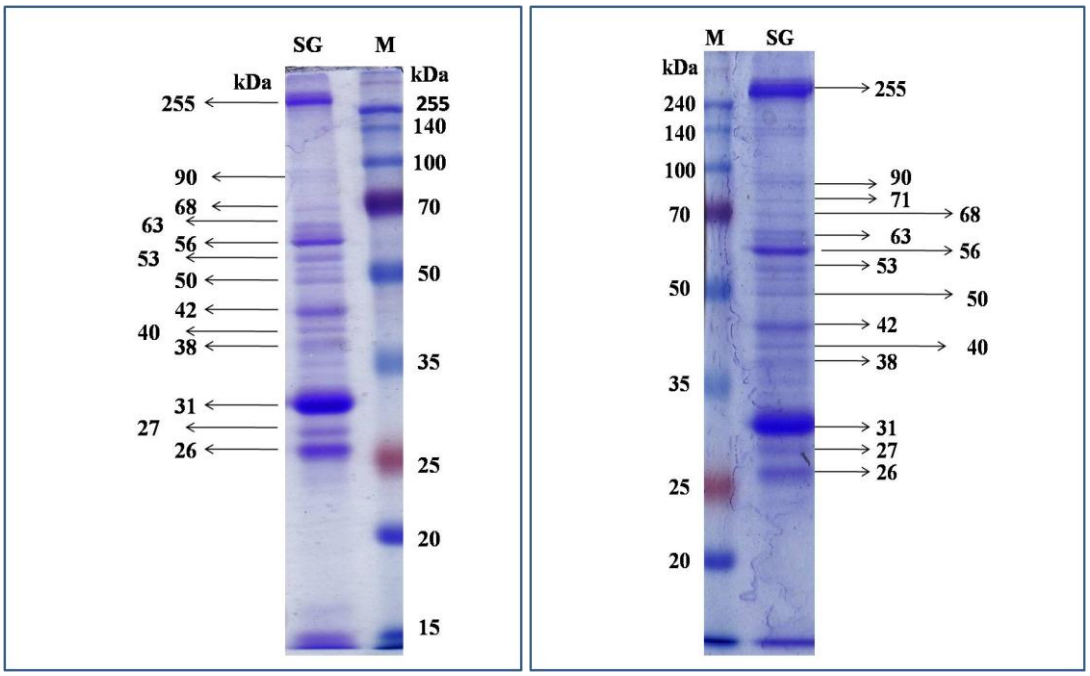


Fig. 1: Single salivary gland dissected from a female *Ae. aegypti* (Nikon stereo microscopy, magnification 8x). The salivary gland is comprised two lateral lobes (2), single medial lobe (1) and the salivary duct (3) connects all salivary gland lobes.

Approximately 13 protein bands were identified with different molecular weights ranging from 26 kDa up to 255 kDa, among them 5 dominant bands of ~255, 56, 31, 27 and 26 kDa were detected (Figure 2). The protein profiles were quite similar between proteins SG from lab. scale cultures and from landing populations. The protein profiles of salivary gland extract (SGE) of *Ae. aegypti* is slightly different from the previously studied by Wonglamchai *et al*. 2010. According to their results,, 13 bands with a molecular weight ranging from 33.5 to > 88.5 kDa were found from SGE of *Ae. Aegypti.* Our study result showed that protein bands higher than 88.5 kDa i.e 90 and 255 kDa, as well as bands with molecular weights lower than 33.5 kDa i.e 26 and 27 kDa. They could be explained by differences genetic structure populations. Since the genetic structure populations depends on human population density (and intensity of insecticidal control) and ecologic characteristics of mosquito ecotopes (Paupy *et al*. 2000). Another results were able to identify several proteins with approximate

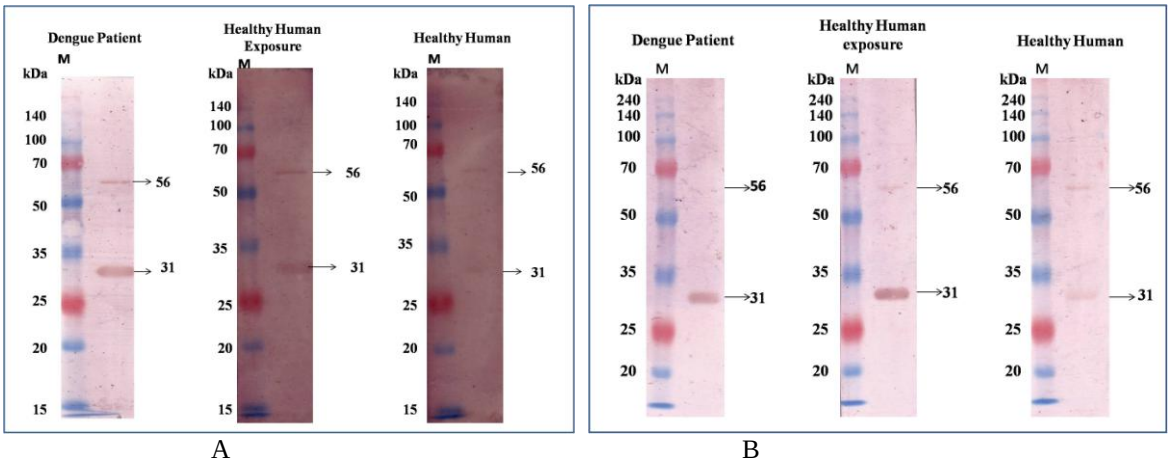
molecular weights ranging from 30 up to 68 kDa by 10% PAGE (Williams *et al*. 2012), however the samples were separated under non denaturing condition. The different in choice of methods is very likely able to give different results in protein profile as well.



A B

Fig. 2: Protein profile of SGE *Ae. Aegypti*. Sample from Lab. scale cultures (A), and from landing populations (B), isolation of 100 pairs SG *Ae. aegypti* (M) marker.

Two protein bands was identified cross-reacted with sera samples from both, DHF patient and healthy people in endemic area i.e. protein with MW of 31 kDa and 56 kDa. Sera sample from individuals living in non endemic area (sub tropical country) and who have never traveled to tropical countries, did not show an immunogenic reaction with SGE of *Ae. Aegypti*. These individuals were very likely never exposed to *Ae. aegypti* bites (Figure 3). These results indicate that people living in endemic areas have specific antibodies that are not found in healthy non-exposed persons and in infants. This can be explained by the following facts. Some salivary proteins are immunogenic and can initiate a specific antibody response (Remouse *et al*. 2006). The development of this natural specific antibodies response in people living in endemic area is due to frequent exposed by saliva (Cornelie *et al*. 2007). Their results showed that children in a malaria endemic area had developed a spesific IgG response against several proteins of *An. gambiae* saliva. A similiar result was also supported by research of Pradines *et al*. 2007, the development of antibody response against *An. gambiae* and *Ae. aegypti* saliva increased significantly in travelers transiently exposed to vector bites in tropical area.



A B

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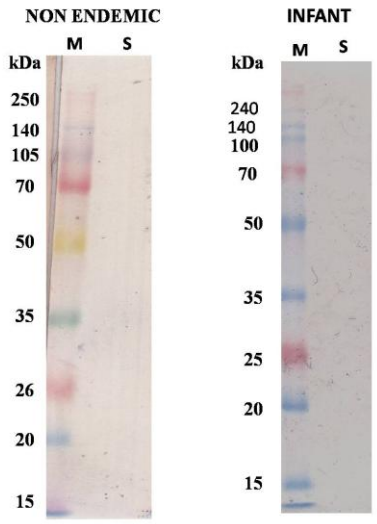

C

Fig. 3: Two immunogenic proteins of salivary gland extract (SGE) *Ae. aegypti* were identified i.e 56 and 31 kDa**,** which is result of cross reacted proteins SG *Ae. aegypti* of lab. scale cultures and from landing populations with 3 different kind of sera samples from endemic area. (A) SGE Lab. scale cultures, (B) SGE from landing population , (C) negative control, (M) Marker.

The specific proteins of 56 and 31 kDa that were detected in this study (Fig. 3), were related to the immune response against SGE of *Ae. aegypti*. The immunogenic proteins 56 kDa SG *Ae. aegypti* has not been reported so far. Whereas the 31 kDa protein is very likely a member of aegyptin group family of the 30-kDa allergen that are very rich in the amino acids glycine, glutamic acid and aspartic acid. Aegyptin has been reported to act as an anti-haemostatic activity protein and play important role in facilitating blood feeding process. It indirectly also plays significant role in enhancing pathogen transmission from vector to host and was also proven as an allergen. Aegyptin will recognize and bind to specific platelet glycoprotein VI (GP VI), integrin α2β1 and VWF (von Willebrand factor) so that the interaction between platelets and collagen will be prevented. Aegyptin is acting as specific ligand for collagen and inhibits platelet activation and thrombocyte aggregation. This collagen is protein matrixes that plays a central role in the process of primary hemostasis and platelet activation that will trigger and stimulate the formation of thrombin (Calvo *et al*. 2007). However, further characterization of the proteins will be very crucial to precisely identify those immunogenic proteins.

According to some studies on transcriptomic and proteomic analysis of salivary proteins from female *Ae. Aegypti*, 120 SG proteins have been identified and 15 of them were identified as secretory proteins involved in the blood feeding process. Some secretory proteins have been described as proteins that can modulate immune respons. These are members of the D7 protein group, adenosine deaminase (ADA), purine hydrosilase, apyrase, and 30 kDa allergen (probably Aegyptin). (Almeras *et al*. 2010). Salivary Proteins from *Ae. aegypti* have been identified as allergens including apyrase protein (68 kDa), α-glucosidase (67 kDa), D7 protein (37 kDa), 30 kDa protein (Peng and Simons, 2004). Since ~ 56 and 31 kDa proteins from this study were immunogenic proteins which is very specific in modulating immune respons from people living in endemic areas, therefore identification of these proteins especially the new 56 kDa and analysis of their activity related to Degue infection, will be a crucial step to investigate their role in Dengue transmission.

Conclusion:

Two immunogenic proteins of salivary gland extract (SGE) *Ae. aegypti* are 56 and 31 kDa, they were able to cross-react with sera sample from people living in endemic area. Those two proteins of salivary gland seem to be important for infectivity of Dengue transmission since they were only found in people living in endemic area.

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Conflicts of interest:

The authors declare that there are no conflicts of interest.

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