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Title

Future prospect of mosquito salivary components as novel target for vector based vaccine against Dengue: molecular characterization of immunomodulatory protein from salivary glands of *Aedes aegypti*

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Dengue Fever (DF) Virus-based Vaccine development showed a relatively slow progress because it should induce protection against the 4 serotypes of Dengue Viruses and there is a very limited adequate animal model for dengue virus infection. In the last decade, new approach in vaccine development for arthropode-borne diseases is using salivary vector components. This approach based on hyphotesis that arthropode vectors saliva contains vasomodulator and imunomodulator proteins that could enhance pathogen infection. Therefore, it should be possible to control pathogen transmission by vaccinating the host against the molecule(s) in saliva that potentiate the infection, thereby blocking the enhancing effects of saliva and thus preventing the pathogen from establishing infection in the host (Transmission Blocking Vaccine, TBV). However, specific component as a potential target for TBV in *Aedes aegypti*, as major vector for DF has not yet been identified so far. This paper wanted to elaborate the immunogenic components from Salivary Gland (SG) of *Aedes aegypti* as potential immunomodulatory protein. We have characterized 2 immunogenic proteins that are only recognized by healthy people living in endemic area and not by people from non-endemic area. They have molecular weight of 31 & 56 kD. Further molecular characterization by Mass-Spectrophotometry of those proteins showed that 31 kDa and dan 56 kDa have high similarity with D7-Protein Family (Odorant Binding Protein) & Apyrase, respectively. These proteins have very important role in vector blood feeding process. This also supported by the strong immunogenicity of 31 kDa against human sera in healthy people as well as Dengue patients. The apyrase activity of 56 kD protein has also been proven in this research. **Keywords**: TBV, *Aedes aegypti*, DF, Salivary Gland, Immunomodulators

INTRODUCTION

There are 2 major mosquito borne-diseases in Indonesia i.e. Malaria & Dengue Fever. Although malaria has been virtually eradicated from Indonesia, it is currently recognized as a serious re-emerging threat to public health. Anti-malarial drug resistances as well as vector resistance against insecticides are major public health problems which hinder the control of *malaria* (e.g. Yadouleton *et al*. 2010). Dengue Fever (DF), caused by infection with dengue virus (DENV), is not a new disease. Today, Dengue is considered as one of the most important arthropod-borne viral diseases in humans in terms of morbidity and mortality. However, life-threatening dengue cases are mostly occurred in west pacific and south east Asia such as Philipina, Thailand and Indonesia (Gubler 1997). In Indonesia, DF cases were first observed in 1968; since then, the incidence has been constantly increasing and the disease is now one of the principal causes of child lethality. It is still characterized as ”Kejadian Luar Biasa” (English= extra ordinary cases), at several provinces. The highest incidence of Dengue was occurred from 1998 – 2000 at Surabaya – East Java Province. The highest proportion of death in DF cases during 1999 – 2000 was associated with children in age of 5 – 9 y.o. (Soegijanto 2004). DKI Jakarta as the capital city, was province with highest cases of DF i.e 14.071 with case fatality rate (CFR) 0,42% in 2003. In 2005, there was increasing DF incidence of up to 23.466 cases with CFR 0,34% (Daniel 2008).

Despite long-standing chemotherapeutic intercession and vector control programs, Malaria as well as Dengue Fever outbreaks are always reported each year. This condition distinguished the quest for causative therapy as extraordinarily daunting. Therefore, development of a vaccine could be a more efficient strategy to overcome the epidemic. In the last decade, new approach in vaccine development for arthropode-borne diseases is by using salivary vector components. This approach based on hyphotesis that arthropod vectors saliva contains vasomodulator and imunomodulator proteins (e.g. Sack & Kamhawi 2001, Titus *et al.* 2006). The vasomodulatory factors in arthropod saliva help the vector to obtain a blood meal. There are 2 hypotheses concerning the function of imunomodulatory factor in saliva of mosquitoes. Many reports showed that salivary imunomodulators could enhance pathogen infection (e.g. Donovan *et al.* 2007) (1). However, there is also evidence that saliva appeared to directly protect dendritic cells from infection *in vitro* in Dengue (Ader *et al.* 2004) (2). In relation with the first case, it should be possible to control pathogen transmission by vaccinating the host against the molecule(s) in saliva that potentiate the infection, thereby blocking the enhancing effects of saliva and thus preventing the pathogen from establishing

infection in the host. In case of second condition, it could be use directly to protect host cells from infection of transmitted pathogens. These hypotheses lead into new field of research that examine these salivary factor especially the imunomodulatory factor to serve as target to control pathogens transmission i.e. Transmission Blocking Vaccine (TBV) or as also known as Mosquito Stage Vaccine (Ramirez *et al.* 2009). However, specific component as a potential target for TBV in mosquitoes i.e. Anopheles as well as *Ae (Ae) aegypti* has not yet been identified so far. Therefore, exploring salivary components of mosquitoes is an important step to localize novel target on TBV development. This paper wanted to elaborate the future prospect of immunogenic proteins from salivary gland of major mosquitoes vector for Dengue i.e. *Ae. aegypti* as potential target for developing TBV.

Immunogenic Proteins from SG of *Ae. aegypti*

Saliva of *Aedes aegypti* could inhibit virus infection in dendritic cells (DC) *in vitro* (Ader *et al.* 2004). Presensitization of DC with saliva could enhance inhibition of infection. Additionally, necrosis of DC cells was decreased after administration of *Aedes aegypti's* saliva in that research. Other effects of that application are increasing of IL-12 p70 and TNF-α production in cell culture. These data suggest protection role of *Aedes aegypti* salivary components. Mosquito bites have also been reported to influence immunity and potentiate viral disease in mouse models (e.g. Limesan *et al.* 2003, Schneider *et al.* 2006), possibly through the modulation of host systemic cytokine responses by the salivary component (Schneider *et al.* 2004). This strategy may be important for the development of vaccines to combat mosquito-transmitted viral pathogens such as Dengue Fever.

Analyzing which protein portions of SG that can be recognized by human antibody, implicating the possibility to find out SG's protein which has significant role in respond to Dengue infection especially those are interacting with healthy human antibody from endemic area. People who are living from endemic area but they do not get the disease even though they have high risk to get infected by the Dengue virus through mosquitoe's bites must have certain immune mechanism which confer this protection. This is relevance with natural resistency which is built by population living at endemic as above explained in case of leishmaniasis. Investigation of specific component that are important for immune response in relation with pathogen transmission was initiated in our research group by using SG of *Ae. Aegypti* (Senjarini, 2013). As seen in Figure 1, we were able to identify specific protein portions of SG from *Aedes aegypti* with molecular weight of ~ 56 & 31 kDa that are able to

cross react only with human sera from endemic area (individual response) as well as population response (Figure 2., Oktarianti *et al*. 2014). These proteins were not appeared in SGE that were cross reacted with human sera who were previously never exposed to Mosquitoe's bites. The ability of this protein to response against human antibody (IgG) of healthy person from endemic area may serve as an indicator for human resistance against Dengue.

In accordance with our current result, further characterisation of proteins in SG from *Ae. aegypti* has to be focused on these specific proteins to be further analysed for their function as also already published previously i.e. putative secreted 30 & 37 kD Protein, Putative DenV binding Protein (54 – 58 kD) and SGS1 Protein (387 kD, consist of 2 fragmen ranging from 120 – 220 kD). These protein have also been identified as a potential proteins conferring immunomodulatory activities (King *et al*. *2011*, Wasinpiyanmonkol *et al*. *2010*). These evidences strongly support the possible development of TBV against DF based on *A. Aegypti* salivary components. Therefore, vaccination by using combination between salivary components that inhibit virus infection and antibodies against salivary immunosupression factors would be an effective and eficient strategy to combat Malaria, DF and possibly also other mosquito borne-diseases in tropical countries such Indonesia.

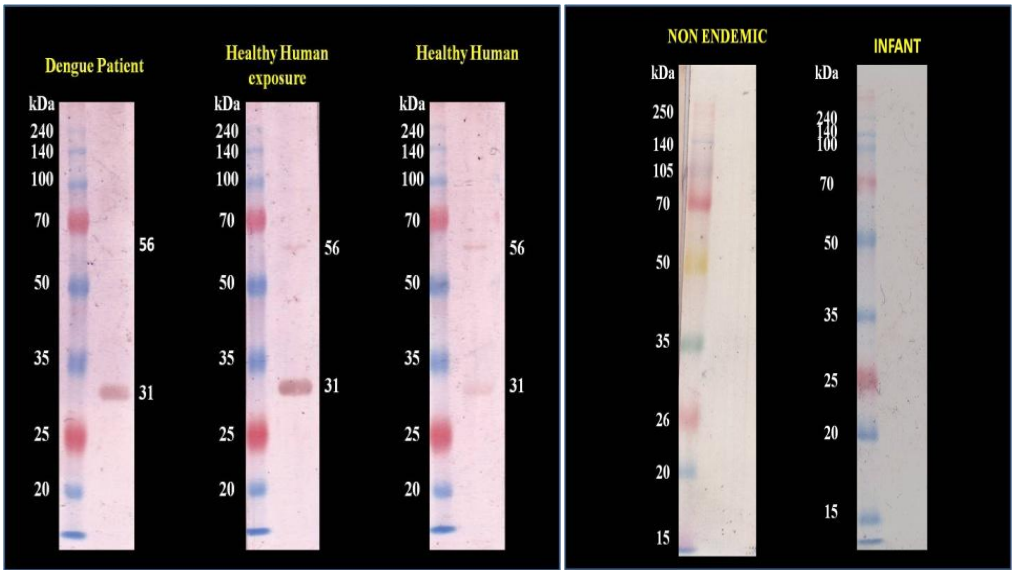

Figure 1. Results of Western Blot analysis showed 2 specific protein from SG *Ae. aegypti* (31 & 56 kDa) was recognized by only human antibody from sera of people living in endemic are (A). Sera from people living in non endemic area were taken from Japanese who were never been traveling to tropical countries (Senjarini, 2013, Oktarianti *et al.* 2014).

Molecular characterization of 37 kDa & 56 kDa from SG of *Ae. aegypti*

Proteomic analysis of two immunogenic proteins with molecular weight of 31 and 56 kDa from SG of *Ae. aegypti* to characterize its molecular properties (Oktarianti *et al.* 2015). These proteins were observed to be very specific in modulating immune response from people living in endemic areas and could also be used as a reliable marker (an epidemiological indicator) for exposures to bites of *Ae. aegypti* (Oktarianti *et al.* 2014). The results were consistent within individual response (Senjarini 2013) as well as pooled response (Ontarianti *et al*. 2014). We identified 13 proteins by LC-MS/MS from the 31 kDa band, including D7 protein (37 kDa salivary gland allergen Aed a2), AAEL003600-PA, long form D7Bclu1 salivary protein, putative 34 kDa, Angiopoietin-like protein variant, 30 kDa salivary gland allergen Aed a 3, annexin, AAEL006417-PA, putative serpin, AAEL003107-PA, AAEL007776-PA, AAEL004338-PA and malate dehydrogenase.

Among the proteins, 5 proteins are secreted proteins involved in blood feeding, including D7 protein (37 kDa salivary gland allergen Aed a 2), AAEL006417-PA, putative serpin, 30 kDa salivary gland allergen Aed a 3, and Long form D7Bclu1 salivary protein. Fifteen secreted proteins were reported to be responsible for the interaction between mosquitoes and vertebrate host during blood feeding. Among them, D7 protein, adenosine deaminase, apyrase, purine hydrolase and 30 kDa allergen have been shown to induce an antibody response and modulating host immune response (Almeras *et al.* 2010).

D7 protein (37 kDa salivary gland allergen Aed a 2) and 30 kDa salivary gland allergen Aed a 3 were responsible for induction of allergic response (Peng & Simons 2004). Pyruvate dehydrogenase and malate dehydrogenase are involved in the citric acid cycle and the expression of both proteins are increased in the SG which infected dengue virus (Chisenhall *et al*. 2014). Annexin has to be capable of binding phospholipids in a calcium dependent and are important in various cellular and physiological processes such as providing a membrane scaffold, which is relevant to changes in the cell's shape . Functions of AAEL003600-PA , Angiopoietin-like protein variant, putative 34 kDa, AAEL003107-PA and AAEL007776-PA have not been reported yet.

Judging from the unused score (81.21), D7 protein (37 kDa salivary gland allergen Aed a2) seemed to be the most abundant protein contained in the 31 kDa band. Unused score is total of peptide not claimed by another protein. However, Almeras *et al.* (2010) have reported that D7 protein has three isoforms with molecular weight of 39, 38, 37 kDa, respectively. Their differences of molecular weight could be explained by differences of

sample population (*Ae. aegypti* from colonies PAEA, *Rockelfeller* and *Formosus*). The D7 protein (37 kDa salivary gland allergen Aed a2) is secreted protein and one of the most abundant in saliva or SG of female blood sucking Diptera. It is related to the odorant binding protein (OBP), which is adapted to binding small ligands and plays a role in binding agonist of haemostasis (Valenzuela *et al*. 2002, Calvo *et al*. 2006, Francischetti 2010).

D7 protein is also known to inhibit the action of biogenic amines such as serotonin, histamine and norepinephrine. It can bind host biogenic amines to antagonize vasoconstictor, platelet-aggregating, a function that might help blood feeding (Ribeiro *et al*. 2007). D7 protein exists in two forms: long form (30-35 kDa), which is found exclusively in mosquitoes and sand flies. On the other hand, the short form (15 kDa) is found in the other insect (Jariyapan *et al.* 2012). Juhn *et al.* (2011) have reported the *in situ* hybridization patterns of 30 genes expressed in the salivary gland of adult female *Ae. aegypti*. Proteins involved in blood feeding such as D7 long form were found to be expressed both in the distal-lateral and medial lobes. The long D7 protein has 10 conserved cysteine residues in its amino acid sequence, with the exceptions that AnSt-D7clu2 and AnAr-D7 do not have the 6th conserved cysteine and that they also lack the terminal 10th cysteine. On the other hand, all of the short D7 proteins have 6 conserved cysteines (Valenzuela *et al*. 2002). The 37 kDa SG D7 protein of *Ae. aegypti* mosquito is responsible for induction of allergic response. The cDNA of Aed a2 has been cloned and sequenced, followed by characterization of the recombinant proteins designated rAed a2 (Wang *et al*. 1999). rAed a2 was identified as allergen and had identical immunogenicity and biologic activity to native Aed a2. rAed a2 binds to the serum IgE antibodies, of which production is induced by native Aed a2. Native Aed a2 is able to bind to the rabbit antibody immunized with rAed a2 (Peng *et al*. 2006). rAed a2 induces a significant increase in IgE and IgG1 and development of positive skin immediate reaction *in vivo* (Wang *et al*. 1999, Peng & Simons 2004).

Putative Serpin belongs to serpin family, which plays a role as an anticoagulant by inhibiting Xa in the blood coagulation process (Ribeiro *et al*. 2007, Almeras *et al*. 2010). Whereas AAEL006417-PA and long form D7Bclu1 belongs to the odorant binding protein (OBP), they were involved in blood feeding. The 30 kDa SG protein (Aed a2) was also indentified. It cause an allergic reaction in human related immune response at the bite site. This protein with very rich glycine, glutamic acid residues was first described in *Aedes* mosquitoes but was also found in *An. Gambiae* (Valenzuela *et al*. 2003, Calvo *et al*. 2007). The 30 kDa SG protein could be aegyptin, which acts as an anti-haemostatic activity protein and plays an important role in facilitating blood feeding process. It indirectly also plays a

significant role in enhancing pathogen transmission from vector to host and was also proven to be an allergen. Aegyptin binds to specific platelet glycoprotein VI (GP VI), integrin α2β1 and VWF (von Willebrand factor) so that the interaction between platelets and collagen would be prevented. Aegyptin acts as a specific ligand for collagen and inhibits platelet activation and thrombocyte aggregation. Collagen, a matrix protein, plays a central role in the process of primary haemostasis and platelet activation that will trigger and stimulate the formation of thrombin (Calvo *et al*. 2007).

The study has identified 7 proteins for the 56 kDa-protein band. i.e Apyrase, AAEL000732-PA, 5'-nucleotidase, AAEL009524, AAEL004739-PA, putative secreted protein and AAEL017349-PA (Table 2). Among them, one protein is a secreted protein and involved in blood feeding i.e apyrase, 3 proteins are involved in metabolism and functions of other 3 proteins are unknown. Based on the unused score, the most abundant protein identified from the 56 kDa band was apyrase (62.754 kDa). Apyrase is nucleoside triphosphate-diphosphohydrolase found in most haematophagous organisms and helps blood feeding by inhibiting platelet aggregation via destroying ADP or ATP. ADP is an inducer which is very important in aggregation platelet processing and ADP released by damaged cells and activated platelets (Fontaine *et al*. 2011). 68 kDa of saliva protein from adult *Ae. aegypti* has been identified as apyrase, it was expressed in the adult female salivary gland (Dong *et al*. 2012). *In situ* hybridization analysis confirms the role of the distal-lateral and medial lobes in the expression of genes involved in blood-feeding, such as salivary apyrase (Smartt *et al*. 1995, Juhn *et al*. 2011). The *Aedes albopictus* apyrase was found in the distal lateral lobes (80%) and also in the medial lobe of the gland (20%). But distribution of the mRNA that encodes the apyrase of female *Ae. aegypti* salivary glands showed that it is present in both distal-lateral and medial lobes (Marinotti *et al*. 1996). While the previous proteomic study has identified 71 kDa and 81 kDa apyrases (Almeras *et al.* 2010), and also 63 kDa apyrase (Doucoure *et al*. 2013).

Currently, only four recombinant mosquito salivary proteins from *Ae. aegypti* were identified as allergens including rAed a 1. Recombinant apyrase (rAed1) inhibits ADP-induced platelet aggregation, which proved that the activity of recombinant apyrase is observed in platelet aggregation assays (Peng *et al*. 2006, Dong *et al*. 2012). Three classes of apyrases were characterized at the molecular level in different blood-sucking arthropods. One is the 5' nucleotidase family proteins, which were cloned from *Anopheles gambiae* and *Ae. aegypti* (Francishetti 2010, Jariyapan *et al*. 2012, Dong *et al*. 2013). 5'-nucleotidase is

involved in nucleotide catabolic process, AAEL009524-PA is involved carbohydrate metabolic process, AAEL017349-PA binds to ATP and is involved in the stress response.

CONCLUSION & OUTLOOK

To initiate eradication of 2 major mosquitoes borne-diseases in Indonesia i.e. malaria and DF, a comprehensive strategy is needed. Chemical intercession and pathogen based vaccine development may not be sufficient to halt transmission. Vector-based strategy could be another alternative, because their target is not only an essential step in the transmission process, but they also block the spread of pathogens, thus preventing rather than treating the illness. The existence of specific protein with MW of 31 & 56 kDa which may serve as important factor to confer Dengue infection resistancy, suggested its important role in human-virus-pathogen interaction. Further molecular characterization of these immunogenic proteins has shown that they are consisting of majority proteins that have important role in term of pathogen transmission into human. To our knowledge, these are the first reports on exploration of salivary glands from Indonesian mosquito's vector. Therefore, to further elucidate salivary role in establishing and/or inhibiting infection, the predominant effectors mechanism in host immune response related to the presence of this protein portion should be further investigated.

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