

BIDANG ILMU: MIPA (BIOLOGI)

EXECUTIVE SUMMARY

HIBAH PENELITIAN TIM PASCASARJANA



**POTENSI *ARTHROPOD-ODORANT BINDING PROTEIN, D7*
dari SALIVA VEKTOR MALARIA *Anopheles maculatus* & *Anopheles aconitus*
dalam MENGHAMBAT PATOGENESIS PARASIT MALARIA**

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Executive Summary

“Development of Salivary Mosquitoes-based
Transmission Blocking Vaccine for Malaria & Dengue”

2014

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Abstract

Mosquito-borne diseases are rampant in most tropical regions of the world, especially rural, forested and coastal areas such as Indonesia. Despite long-standing chemotherapeutic intercession and vector control programs, mosquito-borne diseases exact a heavy burden on human health in Indonesia. Two major public health problems transmitted by mosquito in Indonesia are Malaria and Dengue Haemorrhagic Fever (DHF), causing million clinical episodes occurring annually. Although malaria had ever been virtually eradicated from Indonesia but currently malaria is recognized as a serious re-emerging threat to public health. DHF 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 has been widely observed that saliva of mosquito that transmits disease contains several factors 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. However, specific component as a potential target for TBV in mosquitoes vectors of Malaria & Dengue i.e. *Anopheles* as well as *Aedes aegypti* has not yet been identified so far. There was reduction in parasitaemic rates in mouse model which is previously vaccinated by salivary extract from *A. aconitus*, *A. sundaicus* & *A. maculatus* in our research, indicated the potential role of this vector's saliva to serve as novel target in developing new vaccine targets and novel strategies against malaria. The existence of specific immunogenic proteins 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. 5 immunogenic proteins from *A. sundaicus* have been also characterized i.e. 200, 32, 57, 60, 82 kDa. 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 and molecular characterization of those specific SG's protein should be further investigated.

Keyword: Salivary Gland, Vector, Transmission Blocking Vaccine (TBV), Malaria, Dengue

Introduction

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 (KLB)” 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 (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 potential role of 3 potential malaria vectors in Indonesia i.e. *A. aconitus*, *A. sundanicus* and *A. maculatus* and another major mosquitoes vector for Dengue, *Ae. aegypti* as target models for developing TBV, comparing with other already published vectors.

Methods

Preparation of Sample from Salivary Gland

Mosquitoes reared under strictly identical standard conditions at 28°C and 60% relative humidity at Zoology Laboratory of Biology Department- Faculty of Mathematic & Natural Sciences, Jember University. Female mosquitoes were selected 7–10 days-old after their first blood feeding on rabbit blood for *Aedes* and mice for *Anopheles*. The salivary glands from adult mosquito females were dissected using a fine entomological needle under a stereomicroscope at 4x 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°C until needed. Salivary Gland protein extraction was done by sonication 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.

Immunogenic Proteins of SG from Vectors against Human Blood Sera

Sera sample 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. 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° 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.

Prasitemic rates observation in murine model for Malaria

BALB/c mice aged 6 to 8 weeks were purchased as specific pathogen-free young adults and maintained in accordance with National Institutes of Health guidelines. All mice were male and were age matched for all experiments. Mice were vaccinated with SGE every 2 weeks for 4 weeks (Primary vaccination, Booster I and Booster II). A control group of age-matched mice were only vaccinated by using dilution solution for SGE i.e. PBS in Aluminium hydroxide (v/v=1:1). Murine parasitemia was assessed by using thin-layer blood smears. Smears were fixed in 100% methanol and stained with Giemsa. Mice were exposed to infect with *Plasmodium berghei* (i.p) 2 weeks following the last vaccination. Forty-eight hours post-exposure, blood samples were taken and thin-layer blood smears were made and stained with Giemsa. Subsequently, samples were taken and parasites were quantified each day until mice were euthanized on day 7 postinfection. For the determination of parasitemia levels, 1,000 cells from each sample were counted.

Results & Discussion

Reduction of Malaria Parasitemic Rates by Anopheles Salivary Components

The role of vector salivary components to mediate infection is relatively a new concept in term of Arthropod borne-diseases. This is very important since mortality and morbidity of infectious diseases transmitted by blood sucking arthropods (haematophagus) have been always increasing in the last decade (Gubler 1998). Additionally, there is co-evolution function between vectors and their transmitted pathogen i.e. salivary vector plays actively roles in homeostatic, inflamatic and its vertebrates host immune responses (Ribeiro 1995). Mosquito’s salivary components are immunogenic i.e. inducing strong immune response for example swelling and itching that accompanied mosquitoes bites (Peng & Simon 2004). The host immune response against Mosquito’s saliva could decreased infectivity of transmitted pathogens (Belkaid *et al.* 1998), therefore population living at endemic of leishmaniasis sites showed natural resistancy against leishmania parasites (Davies & Gavgani 1999). This has been also proven in murine model for Leishmaniasis. Prior exposure of mice to bites of uninfected sand flies conferred powerful protection against *Leishmania major* that was associated with a strong delayed-type hypersensitivity response and with interferon- γ production at the site of parasite delivery (Kamhawi *et al.* 2000). The hyphotetical mechanism for those process was explained as natural immunity mediated by Th1 that has protective properties and contains antibodies against sandflies’s saliva. Mosquitoes bites have shown similar effects in animal models through cytokines systemic response in Host (Schneider *et al.* 2004). It could be concluded recently that these imunomodulatory components have immunosupresive properties towards effector cells such as macrophage, T lymphocytes, B lymphocytes, Natural Killer cell and granulocytes (Andrade *et al.* 2005). Another evidence showed that repeated exposure to bites from uninfected *Anopheles stephensi* skewed the immune response towards a T-helper 1(Th1) phenotype as indicated by increased levels of interleukin-12, gamma interferon, and inducible nitric oxide synthase which was followed by reduction of parasitemic rates in Mouse Model (Donovan *et al.* 2007). Another mechanisms related to this protection were very likely due to immunogenicity of salivary protein from *Anopheles* which have been proven from *A. gambiae* (Poinsignon *et al.*,

2009), *A.arabiensis* and *A. funestus* (e.g. Lombardo *et al.*, 2009; Rizzo *et al.*, 2011) as well as from *A. barbirostris* (Jariyapan *et al.* 2012).

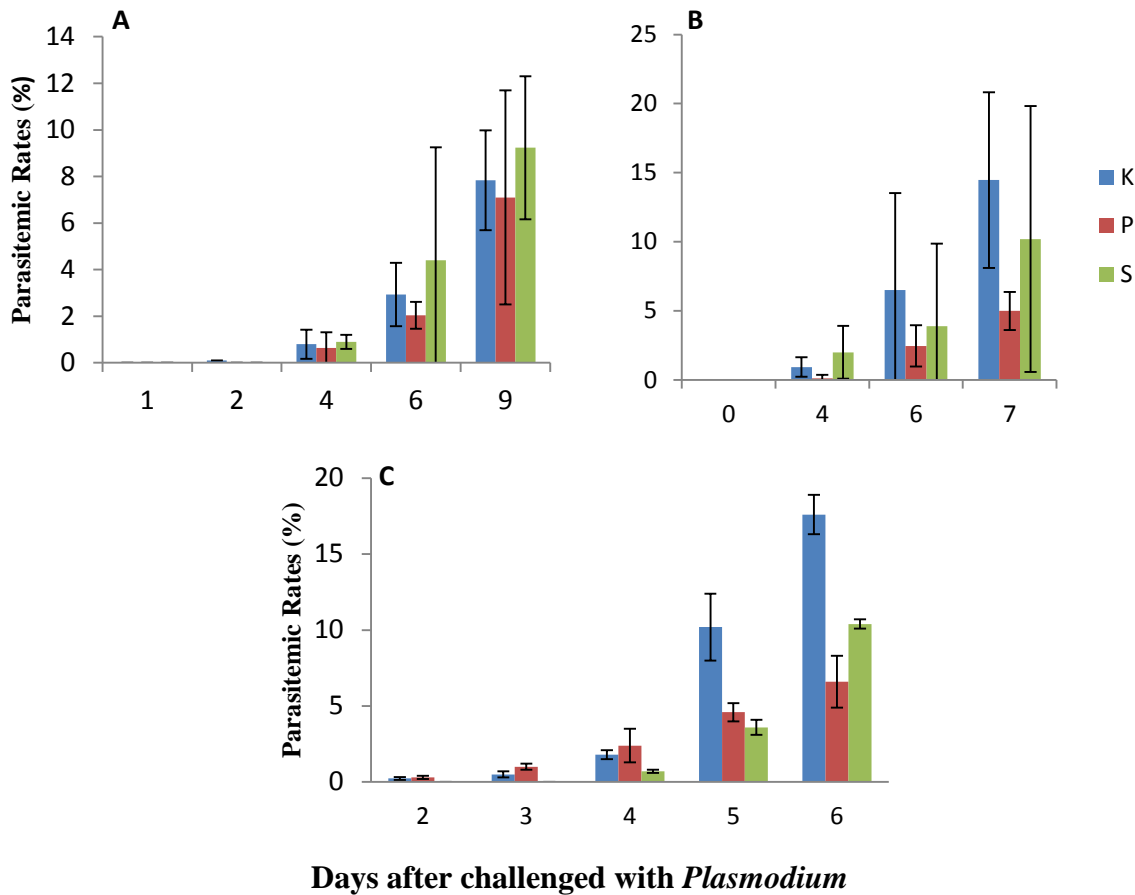


Figure 3. Average of parasitemic rates (%) in murine model: Control (K), vaccinated with SGE from Pelet (P), and vaccinated with SGE from supernatant (S) from *A. maculatus* (A), *A. aconitus* (B), dan *A. sundaicus* (C). K= Control, P= Pellet from Salivary Gland Extract (SGE) & S=Supernatant from SGE. Numbers represent days after exposing murine model with *P. Berghei* (Senjarini *et al.* 2013)

Our results showed that repeated exposures of salivary gland (SG) extract from 3 potential malaria vectors in Indonesia i.e. *A. aconitus*, *A. sundaicus* and *A. maculatus* were able to reduce parasitemic rates in murine model for malaria (Figure 1.). This reduced parasite burdens suggested the function of host immune response towards saliva to reduce infectivity of transmitted pathogen. Those data indicated that mosquito salivary components may be served as

a nonspecific potentiator whose effect to induce a Th1-biased environment that is known to be effective against malaria infection. Therefore, addition of *Anopheles* salivary components to antimalaria vaccines may be a viable strategy to improve vaccine's efficacy. Further studies on molecular characterization of component in salivary gland that is responsible for this process is needed to understand salivary role in blocking transmission of malaria parasites.

Immunogenic Proteins from SG of *Aedes aegypti* & from *Anopheles sundaicus*

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 mosquito'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* (Oktarianti *et al.* 2013). As seen in Figure 2., 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). These proteins were not appeared in SGE

that were cross reacted with human sera who were previously never exposed to Mosquitoes' 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 immunosuppression factors would be an effective and efficient strategy to combat Malaria, DF and possibly also other mosquito borne-diseases in tropical countries such Indonesia.

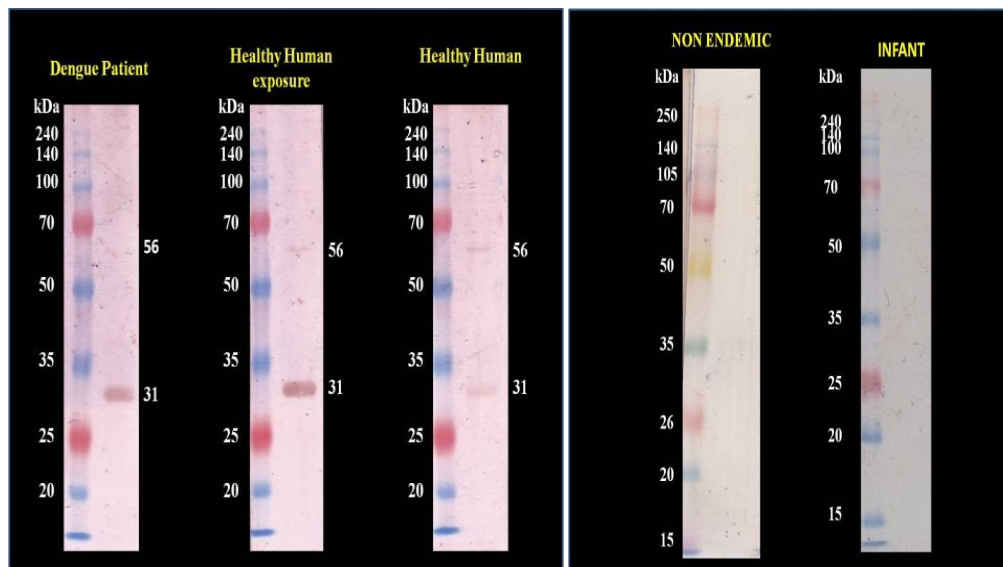
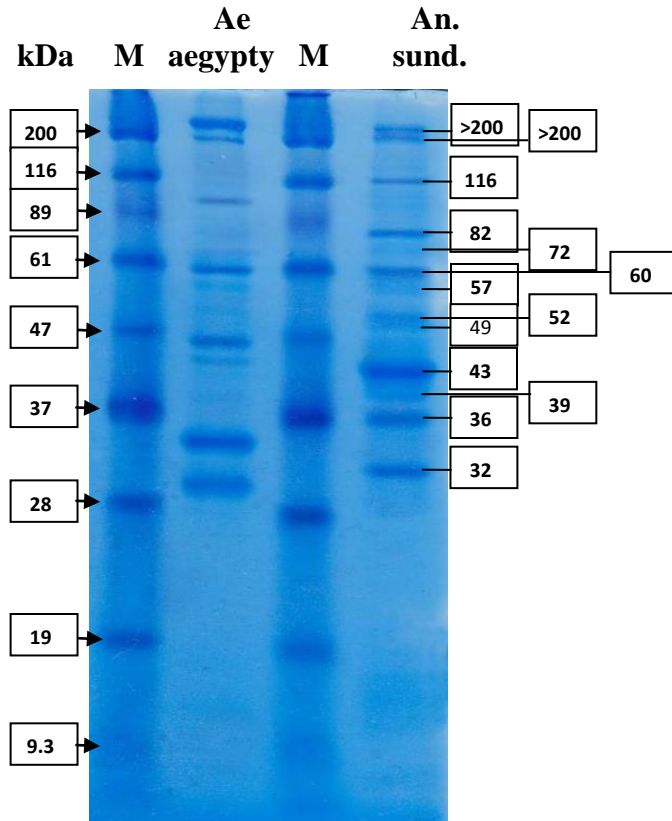
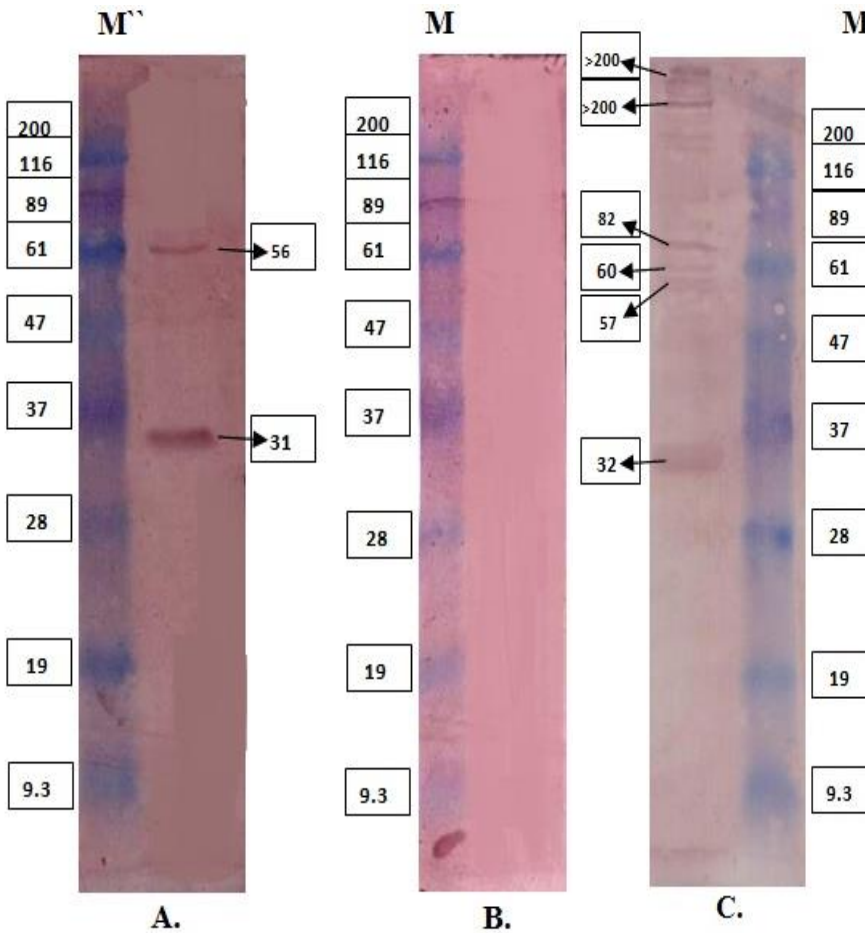


Figure 2. Results of Western Blot analysis showed 2 specific protein from SG *Ae. aegypti* (31 & 56 kDa) was recognized by only human antibody from sera of people living in endemic area (A). Sera from people living in non endemic area were taken from Japanese who were never been traveling to tropical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

The same analysis was conducted to SG of malaria vector *A. sudaicus*, out of 13 protein bands appeared after SDS PAGE analysis (Figure 3), only 5 proteins were immunogenics (Figure 4). These immunogenic proteins only recognized by human sera from people living in endemic area but not from non endemic area, implicating its specificity for endemic human immune response.



Gambar 5.5. Hasil visualisasi SDS-PAGE, (SG) protein 10 pasang kelenjar saliva *An. sudaicus* betina; dengan marker (M) Pre-strain Intron. (scan: Cannon MP 250).



Gambar 5.6. Hasil *Western Blot*: (A) 2 protein imunogenik yaitu 31 kDa dan 56 kDa (Anti human IgG sehat endemik DB); (B) orang sehat non endemik; (C) 6 protein imunogenik dari ekstrak kelenjar saliva *An. sundaicus* 32, 57, 60, 82, > 200 kDa (Anti human IgG sehat endemik malaria, M = marker (Pre-strain Intron)).

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 targets 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. Reduction in parasitaemic rates in mouse model which is previously vaccinated by salivary extract from *A. aconitus* & *A. maculates* in this research, indicated the potential role this vector’s saliva to serve

as novel target in developing new vaccine targets and novel strategies against malaria. Many reports suggest that any measure which limits parasite densities will reduce the morbidity and mortality associated with malaria infection (McErroy *et al.* 1994). 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. 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 and molecular characterization of those specific SG's protein should be further investigated.

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