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Research Article

IgG IMMUNE RESPONSE AGAINST SALIVARY GLAND PROTEIN EXTRACT OF DENGUE VECTOR *Aedes aegypti*

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ABSTRACT. The implication of *Aedes aegypti* (*Ae. aegypti*) salivary components on the host immune system remains a crucial factor to be revealed for evaluating the risk of dengue infection. The salivary gland of mosquitoes has been known to contain several biological components that facilitate blood-feeding and contribute to successful pathogens transmission. These processes are mediated by the antigenic and immunogenic molecules inside the salivary cocktails. During the blood-feeding, mosquitoes injected the saliva and hosts will develop immune responses as a counter-attack against salivary components. Several studies showed that the evaluation of antibody responses towards arthropod-vector saliva could be a biological indicator to estimate the vector's exposure. Sensitisation of *Ae. aegypti* Salivary Gland Protein Extract (SGPE) were used to clarify mosquito feeding's implication to host humoral immune response (IgG). Enzyme-Linked Immunosorbent Assay (ELISA) was used to analyse IgG quantitatively from sera sample of the murine model, i.e. BALB mice. Results showed that elevated IgG levels were in accordance with the increasing concentration of SGPE as well as longer time of exposure. This result indicated that mice immune response had been modulated by *Ae. aegypti* salivary components. Therefore, we could assess *Ae. aegypti* salivates exposure by analysing IgG quantitatively as potential biomarkers for vector bites.

Keywords: *Blood feeding, IgG, immunogenic, pathogen transmission, sera.*

INTRODUCTION

The incidence of mosquito-borne diseases, such as dengue infection, has grown dramatically in the last few decades. The diseases are caused by dengue virus (DENV) with four distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4), a member of Flaviviridae family and Flavivirus genus [1, 2]. Dengue virus is transmitted by *Ae. aegypti* as the primary vector, accompanied by *Ae. albopictus* as the secondary vector [3]. Dengue infection occurs when an infected mosquito takes up host-blood, thus inoculating its virus-containing saliva [4].

The correlation between host-vector interaction is a decisive stage in pathogen transmission. Dengue virus as pathogens goes through mosquitoes midgut and ultimately reaches the salivary gland. The salivary glands of the mosquito vector are the last deposit organ for virus propagation before being transmitted to the host's body [5]. Several studies stated that the saliva of arthropod vectors has a vital role in blood feeding and determines the success of pathogens transmission [6, 7, 8]. The morphological and biochemical studies on the salivary gland of arthropod vectors have been widely conducted [9, 10, 11]. In addition, transcriptomic and proteomic analyses of saliva from arthropod have also been carried out [12, 13, 14, 15, 16]. The saliva of arthropod vectors

contains an anticoagulant, anti-inflammatory, and immunogenic factors [5]. They also have dramatic effects on the host's immune system.

As a homeostatic mechanism, the host's body has developed immune responses to eliminate foreign components, including mosquito salivary components. The immunogenic components of vector saliva are responsible for triggering several host-immune responses [17]. These responses include haemostatic pathways, hypersensitivity, inflammatory, antigen-presenting mechanism, activation of cell-mediated, and humoral immune responses [9, 18]. Eventually, vector bites will induce the host to produce anti-vector antibodies as counter-attack response. The impact of vector saliva on the host-antibody responses is dependent on the duration and intensity of exposure [9]. Seemingly, repeated exposure of vector saliva has induced the host-immune system to confer protective defences against pathogens invasion. This notion refers to the study in *Phlebotomus papatasi*, which contends that saliva exposures can mediate resistant mechanism in the animal model to Leishmania Mayor [19, 20]. Several kind of research discovered natural antibodies produced in response to vector saliva from people living in the endemic area [21, 22]. These reports suggest that antibody response can be a basic understanding for evaluating the exposure to arthropod-borne diseases, which eventually generate the risk of pathogens transmission.

However, a reliable marker for evaluating host-vector contact has not been identified. This research aimed to evaluate IgG antibody response in the animal model after sensitization with *Ae. aegypti* Salivary Gland Protein Extract (SGPE) to shed lights on host immune response towards the exposure of vector's saliva. Furthermore, it is projected to be an essential initiative for finding a definitive biomarker of *Ae. aegypti* exposure related to dengue infection.

MATERIALS AND METHODS

Rearing of Ae. aegypti

Ae. aegypti mosquitoes were bred from larvae, collected from water containers in the field. Adult *Ae. aegypti* mosquitoes were kept in insectarium at 28 °C and 60% relative humidity at Animal Care Unit, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Jember. Mosquitoes were given a cotton wool pad soaked in 10% sucrose solution as well as wistar rat for blood meals. Adult mosquitos were taken out of cage using an aspirator and put into a cup for preparing salivary gland dissection.

Salivary Glands Collection

The salivary gland was isolated from adult female *Ae. aegypti*. The mosquitoes were immobilized by cooling them on ice for 15 minutes and placed on the glass-sterilized object with 70% ethanol. The immobilized-mosquitoes were dissected by the microdissection method using a fine entomological needle under stereo-microscope. The salivary glands were collected in 1 mM Phenyl Methyl Sulfonyl Flouride (PMSF) in Phosphate Buffer Saline (PBS) (Sigma, USA) and kept at -20 °C.

Extraction and Quantification of SGPE

Salivary glands were added with lysis buffer containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 10 mM NaCl, 2 mM EDTA-NaOH, and 1% Nonidet P-40 (Sigma, USA) [23]. The samples were then homogenized using micropestle and sonicated using water-sonicator

for 30 minutes. The supernatant was separated by centrifugation at 12.690 rpm for 15 minutes at 4 °C. The SGPE was filtrated from this supernatant by using ultrafiltration (10 kDa Molecular Weight Cut Off Microcentrifuge) (Corning, USA) and centrifuged at 10.000 rpm in 4 °C by repeating the procedure for several times, so the concentration attained higher density. The quantification of *Ae. aegypti* SGPE was determined by Bradford assay (Sigma, USA) using various concentrations of Bovine Serum Albumin (BSA) (25 to 2000 µg/mL) (Sigma, USA) as standard. The concentration of SGPE proteins was measured at 595 nm [24]. SGPE protein was kept at -20 °C until further used.

Exposure of Animal Model to Ae. aegypti SGPE and Sera Preparation

In this study, we used SGPE as an antigen to stimulate the IgG immune response of the animal model. This treatment is carried out to adjust the mosquito saliva exposure in the natural condition of blood-feeding to the host. We used female BALB-C mice aged 3 to 4 weeks and 25-30 mg of body weight, as the animal model in this study. The animals were divided into four groups which were treated with 0.2 µg/µl, 0.4 µg/µl, 0.6 µg/µl SGPE diluted in PBS respectively, and control. *Ae. aegypti* SGPE was injected by intradermal once per 2 weeks for 8 weeks. Blood samples were collected from sinus orbitalis every 2 weeks after injection. Serum preparation was performed by centrifugation at 3200 rpm for 15 minutes at 4 °C. The supernatant was stored at -20 °C.

Evaluation of IgG Levels by ELISA

Concentrations of IgG from the animal model were measured by Enzyme Link Immunosorbent Assay (ELISA). Microplate with ninety-six wells was coated with 50 µl *Ae. aegypti* and several wells without SGPE were seeded with PBS as control. The microplate was incubated for 12 hours in 4 °C. The solutions were removed, and microplate was washed by 200 µl PBST for 3 times. Then, the microplate was added with 50 µl mice-sera (1:100) as primary antibody in each well and incubated for 2 hours in 37 °C. After that, the solutions were removed, and microplate was washed by 200 µl PBST for 3 times. Then, the microplate was added with 50 µl Goat Anti-Mouse IgG (KPL, USA) (1:5000) as a secondary antibody in each well and incubated for 1 hour at 37 °C. The solutions were removed, and microplate was washed by 200 µl PBST for 3 times. Subsequently, the microplate was added with 50 µl Tetramethylbenzidine (TMB) (Sigma, USA) in the darkroom and incubated for 12 hours. The solution containing 50 µl H_2SO_4 (Sigma, USA) was added in each well to stop the reaction. The absorbances were measured by microplate reader (BIO-RAD, USA) at 450 nm wave lenght.

Statistycal Analysis

The absorbance value was analyzed by ANOVA using SPSS16 to determine the significant difference of IgG levels between each concentration of treated mouse group and negative control group. We carried out 4 types of treatment on 20 mice (5 individuals in each treatment). The control and treatment value of each individual (1^{st}, 2^{nd}, 3^{rd}, 4^{th}, and 5^{th}) then compared. The value was interpreted to be same as IgG level that was successfully bound to the salivary gland protein extract from *Ae. aegypti*. Based on ELISA principle, it should be noted that he higher the absorption value, the higher the level of IgG.

RESULTS AND DISCUSSION

Salivary Glands of Ae. aegypti

Ae. aegypti is the primary vector for dengue and is commonly found either in subtropical or in the tropical regions, such as in Southern USA throughout China and into Australasia, including Indonesia [25]. It is well known that only female *Ae. aegypti* is hematophagous. However, both male and female *Ae. aegypti* can also take sugar for feeding [10, 24]. Specifically, female *Ae. aegypti* requires vertebrate-host blood for nutrition, egg development, and survival [27]. Relevant to our result on the salivary glands (SG) are morphological characteristics (Fig. 1).

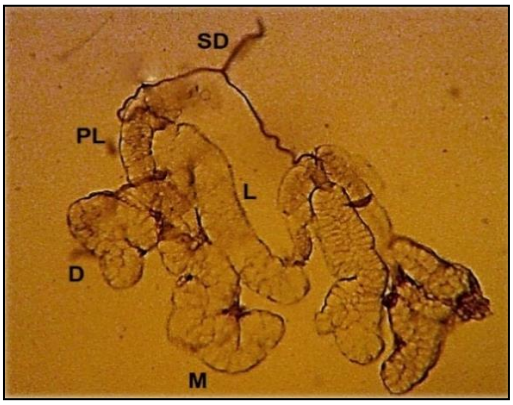

Fig. 1. *Salivary gland of female Ae. aegypti (taken by stereo microscope at 40x magnification, camera: opti lab (Olympus, USA)); (M) Medial lobes, (L) Lateral lobes, (SD) Salivary Ductus, (DL) Distal Lateral lobes, and (PL) Proximal Lateral lobes.*

Ae. aegypti's SG are paired organs, and each gland is composed of three lobes, i.e. two Lateral Lobes and one Medial Lobe (ML). The lateral lobes are further defined into two regions, Proximal Lateral lobes (PL) and *Distal Lateral* lobes (DL) [28]. The salivary gland of female *Ae. aegypti* has dual roles in blood and sugar feeding [15]. Proximal Lateral lobes synthesize enzymes involved in sugar meals, while Medial lobes and Distal Lateral lobes produce molecules related to blood meals [10, 16].

IgG Levels in Mice BALB-C after Sensitization with Ae. aegypti SGPE

Dengue Fever (DF) and Dengue Haemorragic Fever (DHF) are major public health threats in tropical and subtropical countries [21]. There are 50 billion cases of dengue infection reported, and numerous countries have reported that the cases are on the rise, including Indonesia [29]. The first reported DHF epidemic areas in Indonesia are Surabaya and Jakarta [30]. Nowadays, the disease is endemic in urban areas and has been spread to rural areas as well. Therefore, a meticulous approach for evaluating such risks is urgently needed in endemic areas.

An epidemiological study contends that exposure to mosquito bites trigger anti-mosquito SGPE antibodies [31]. Additionally, antibody response has particular specificity and recognizes species-specific antigen from vector saliva [32]. The host's antibody response to mosquito SGPE is short-lived [32]. Furthermore, another study has confirmed the reduction of antibody anti-mosquito SGPE in the host when the exposure

to that mosquito is not sustained [34]. The vertebrate-host antibody response to *Ae. aegypti* SGPE was investigated with various injected SGPE concentrations and also across different time intervals. The IgG quantification was done on individual serum from each group and also pool serum for representing IgG in each group population. The results on individual serums show the specific response of each individual, while the results on the group show the general results of the whole of individual serum mixed in one sample. The results on individual serum showed that, compared to the control, there was an induction of IgG production in animals model after sensitization with *Ae. aegypti* SGPE. Statistical analysis show that there was significant difference of IgG levels between each concentration and control in 2nd individual sera. While in the 1st, 3rd, 4th, and 5th individual sera, there were no significant difference of IgG levels between each concentration and control of treated mice. These individual responses represent the comparison value of each treatment and control in each mouse. Based on these results, although there were different variations in each individual IgG response and each SGPE concentration, there was a tendency to increase IgG production during the experimental period (Fig. 2). The graph shows that there were consistent increases in IgG levels although it is not significant.

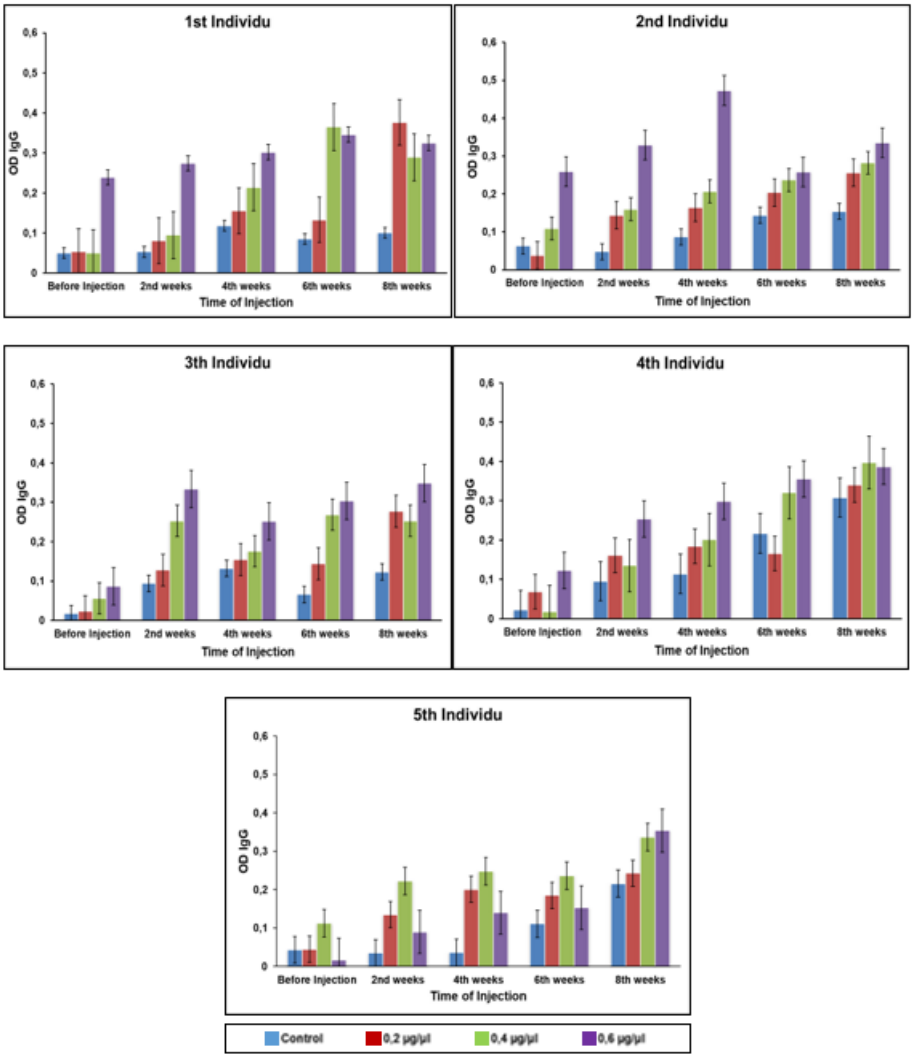

Fig. 2. *Individual IgG response to Ae. aegypti SGPE with various concentrations.*

Varied IgG responses in model mice were assumed to result from genetic factors, nutritional absorption, and also hormonal system [35, 36]. The gene expression that encodes cytokines, including IgG gene, may vary across individual responses [37], influencing each individual response to antigen sensitization. The nutritional conditions can also affect cellular and humoral immune responses. This is also the case of amino acid and vitamins deficiency, decreasing its antibody production [36]. IgG levels in individual response can also be influenced by hormonal factors. Interaction between the reproduction system and an immune response is linked with sexual hormones and the receptors on immune cells [38]. Female mice tend to have fluctuating hormone levels, i.e. progesterone and estrogen. Progesterone is can decrease antibody production [39], while, estrogen involved in elevating antibody production by activating B-cells pathway, including IgG [40, 41]. Still, the comparable results between control and all treated mice in our results showed the predominant effector for the increased IgG in the murine model, which is very likely to be induced by the SGPE of *Ae. aegypti*.

Fig. 3 showed that IgG levels in all groups of animals model raised up significantly, which had been considered a result of increased SGPE concentration as well as long and continuous time of exposure. Statistical analysis for those animals shows that there was significant difference of IgG levels in the negative control with 0.4 ug/ml concentration and negative control with 0.6 ug/ml concentration. However, there was no significant difference in negative control with 0.2 ug/ml concentration, 0.2 ug/ml concentration with 0.4 ug/ml concentration, 0.2 ug/ml with 0.6 ug/ml concentration, and 0.4 ug/ml concentration with 0.6 ug/ml concentration in every week. Thus, the different of IgG production in the animal model was affected by the concentration of injected SGPE, time and the frequency of exposures. Therefore, *Ae. aegypti* SGPE exhibits several interesting patterns that a potential biomarker should posses, i.e. sensitivity to the concentration of injected *Ae. aegypti* SGPE which increases by repeated exposures of *Ae. aegypti* SGPE throughout the test period.

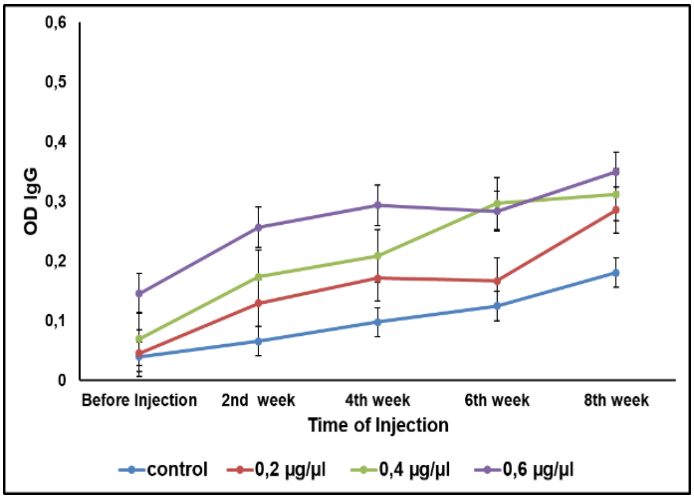

Fig. 3. *IgG levels of Ae. aegypti SGPE according to concentration groups injected (the highest mean of OD value was discovered in 0,6 µg/µl concentration groups).*

The immunological studies stated that in early exposure of *Ae. aegypti* saliva, vertebrate-host will develop Th1 immune response by producing IL-2 and IFN-γ cytokines. In contrary by subsequent exposure, there is a polarization of vertebrate-

immune response towards Th2 immune response with increased IL-4 and IL-10 cytokines [6, 9, 42]. Interestingly, the alteration of vertebrate-immune response from Th1 toward Th2 indicated by up-regulating of IL-4 and IL-10 levels can activate B-cell and differentiate plasma cells that produce specific antibody, such as IgG [40]. Several studies have acknowledged that residents living in endemic areas, either malaria or dengue-endemic areas, usually develop protective immunity to these infections [21, 22, 35, 43, 44, 45]. Our previous study in Indonesia demonstrated that sera from human living in dengue-endemic areas contained specific antibodies sequentially exposed to *Ae. aegypti* saliva [43]. It is known that repeated exposure to vector saliva may positively affect the vertebrate-host immune response. Consequently, IgG response is likely to be a serological marker towards the exposure to *Ae. aegypti*.

CONCLUSION

The results described in this study show that repeated exposure of *Ae. aegypti* saliva has a positive impact on elevating IgG responses. It is also proven that the increase of IgG levels is in accordance with increased concentration as well as longer periods of SGPE. Therefore, in term of host-vector interaction, analysing IgG in host immune response can be used as a biomarker for vector's exposure which is essential for epidemiological study of vector-borne diseases.

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Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: S.W., K.S., Design: S.W., R.O., K.S., Data Collection or Processing: S.W., W.P., Analysis or Interpretation: S.W., R.O., K.S., Literature Search: S.W., W.P., Writing: S.W., K.S.

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