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Advances in Biological Sciences Research

Asmoro Lelono · Muhammad Akbar Bahar · Syubanul Wathon · Kartika Senjarini · Asep Ginanjar Arip · Ramdhan Putrasetya · Beny Andika · Nadhea Ayu Sukma · Bambang Sugiharto *Editors*

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Preface ICOLIB 2021

I am pleased to welcome all of the speakers and participants to the 2021 International Conference on Life Sciences and Biotechnology (4th ICOLIB), which is held from 15 to 16 November 2021, virtually on Zoom meeting. The conference is organized by the Department of Biology, Faculty of Mathematics and Natural Sciences, the University of Jember.

This year the conference's theme is "Towards Sustainable Development: Application of Biosciences to Improve Welfare and Quality of Life". Along with the theme, we have four conference topics; there are Applied Sciences (Agriculture, Biotechnology and Bioinformatics), Basic Sciences (Ecology, Zoology, Botany, and Microbiology), Biodiversity and Bio-conservation, and Health and Medicine (Pharmacy and Medical Sciences). This scientific event provides a platform for researchers, academics, professionals, industries, and policymakers to exchange ideas, share the recent advances and development in life sciences, and can be a valuable place for starting fruitful collaboration, especially in uncovering the potential of biodiversity at the molecular level to biosphere.

This year's conference is also the first time held online due to the global pandemic situation. However, it becomes a blessing in disguise, because the conference becomes accessible to a wider audience and participants from all over the world. The number of participants registered is 223, among them, 170 participants will present their research.

Most of the participants are from Indonesia but also we have participants from Malaysia, Czech Republic, China, Philippines, Pakistan, and India. Besides the participants, the online of this conference also allows us to invite speakers from Netherlands, Germany, USA, and Australia. There will be Prof. A.G.G (Ton) Groothuis from GELIFES Institute, Groningen University Netherlands, Prof Antonius Suwanto from IPB Indonesia, Prof Simon Griffith from Department of Biological Sciences at Macquarie University, Sydney, Dr. Jorge A. Santiago-Blay from the Department of Paleobiology MRC-121 National Museum of Natural History Smithsonian Institution, USA, Prof. Elvira Hoerandl from George-August, Goothingen University Germany, Dr. Kahar Muzakar from Biology Department, Jember University, and Dr. Christina Bauch from Instituto Universitario in Lisbon, Portugal and Groningen University, Netherlands.

The output of this conference will be published in the Atlantis Press Proceeding: Part of Nature in series of "Advances in Biological Sciences Research", the Journal of ILMU Dasar MIPA, and the Journal of Tropical Biodiversity and Biotechnology. Finally, I would like to acknowledge the Board of Jember University which supported this conference. And I also like to thank to Vanadia and DAAD as one of the main sponsors which make this conference possible.

I hope this conference will be fruitful for everyone. We look forward to seeing you all at the next ICOLIB conference.

Asmoro Lelono
Chairman of 4th ICOLIB 2021

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AQ1 All of the articles in this proceedings volume have been presented at the International Conference on Life Science and Biotechnology (ICOLIB) on November 15–16, 2021 at Jember University Indonesia. These articles have been peer-reviewed by the members of the Scientific Committee of ICOLIB and approved by the Editor-in-Chief, who affirms AQ2 that this document is a truthful description of the conference's review process.

1 Review Procedure

The reviews were double-blind. Each submission was examined by two reviewer(s) independently. The conference submission management system was easy chair.

We divided the submission of the ICOLIB participant into two categories, the first is abstract and the second the full manuscript submission. The submissions of the abstract were first screened for generic quality, relatedness to the main topic and suitableness by the editorial team. Based on this initial screening, all of the abstracts would be classified by the main theme, i.e. Applied Sciences (Agriculture, Biotechnology & Bioinformatics), Basic Sciences (Ecology, Zoology, Botany, and Microbiology), Biodiversity & Bioconservation, Health & Medicine (Pharmacy & Medical Sciences). All of the selected abstracts would be presented by their author during the ICOLIB conferences. The committee gave an option to the authors to publish their manuscripts or just presented their work in the ICOLIB conference.

All of the complete manuscripts then follow the review process, the first step was to evaluate the relatedness to the proceedings series "Advances in Biological Sciences Research", scientific quality, novelty and contribution to the science. The second step is checking the similarity using Turnitin to evaluate the textual overlap and detect the possible sign of plagiarism. The third step was to send for peer review by matching each

B. Sugiharto—Editors-in-Chief of the ICOLIB.

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paper's topic with the reviewers' expertise, taking into account any competing interests. However, in some case, we also sent the manuscript to the third reviewer to consider another opinion if the first two reviewers has an opposite decision. A paper could only be considered for acceptance if it had received favourable comments and suggestions from the two reviewers. The recommendations then sent back to the author to address the reviewer's comment. The acceptance or rejection of a revised manuscript was final. In the final steps, all of the manuscripts were adjusted in their layout and some of the technical editing for the pre-print version. This preprint document would be sent to the author for clarification. They also should be sent a statement of the novelty and originality of the study.

2 Quality Criteria

Reviewers were instructed to assess the quality of submissions solely based on the academic merit of their content along the following dimensions. The editorial gave a rubric for a guideline which contains some important aspect related to the quality of the manuscript such as:

1. Pertinence of the article's content to the scope and themes of the conference;
2. Clear demonstration of originality, novelty, and timeliness of the research;
3. Soundness of the methods, analyses, and results;
4. Adherence to the ethical standards and codes of conduct relevant to the research field;
5. Clarity, style, cohesion, and accuracy in language and other modes of expression, including figures and tables.

We have a policy that each manuscript should be reviewed by two reviewers and each reviewer only reviews two manuscripts. The consequences of this policy is that we contact more reviewers, in total we ask 61 reviewer for completing the review process.

3 Key Metrics

Total submissions	118
Number of articles sent for peer review	67
Number of accepted articles	61.
Acceptance rate	51.5%
Number of reviewers	61

4 Competing Interests

Neither the Editor-in-Chief nor any member of the Scientific Committee declares any competing interest.

Humoral Immune Response (IgG) of BALB/c Mice (*Mus musculus*) Post-injection by 56 kDa Immunogenic Protein Extract from the Salivary Glands of *Aedes aegypti* L.

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Abstract. The primary mosquito vector of dengue virus is *Ae. aegypti*, and dengue virus transmitted by saliva mosquito's during blood feeding. The saliva composed of numerous proteins with capacity to disrupt hemostasis or modulate the host immune response. The previous study identified two immunogenic proteins in the salivary glands of *Ae. aegypti* i.e. 56 kDa and 31 kDa. However, the mechanism of host's humoral immune response (IgG) against the 56 kDa immunogenic protein is not known. Therefore, this study was conducted to observe the mice strain BALB C immune response (IgG) against the 56 kDa. The mice were divided into three groups of injection treatments, particularly Group-A (Tris-Cl 0.05M), Group-B (adjuvant), and Group-C (56 kDa immunogenic protein 0.1 μg/μL + adjuvant). Injection was done every two weeks in total six weeks of treatment. The IgG level was measured every two weeks using ELISA method. The study results exhibited the Group-C had higher IgG level than Group-A and Group-B. The IgG level of Group-C gradually increased along the increasing of 56 kDa protein exposures, meanwhile the IgG level of Group-A and Group-B tended to be steady before and after treatment. These results suggested that repeated exposure of 56 kDa immunogenic protein elevated the humoral immune response (IgG) in host.

Keywords: 56 kDa immunogenic protein · Salivary gland · Ae. aegypti · IgG

1 Introduction

Dengue virus (DENV) is a flavivirus with four different serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) [1]. Approximately 390 million people are infected by DENV annually, with highest prevalence in tropical countries [2]. DENV is transmitted to humans by *Aedes spp.* Mosquitos as vectors. *Ae. aegypti* and *Ae. albopictus* are dominant vectors for DENV transmission. *Ae. aegypti* is the main vector and *Ae. albopictus* is the secondary vector [3]. *Ae. aegypti* is adaptable to live in urban areas. It can breed easily in artificial or natural containers near to human residences in urban areas. *Ae. aegypti* has blood-meal preference on human, therefore it can facilitate the transmission of arbovirus

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to human easily. In contrast, *Ae. albopictus* predominantly lives in vegetated and rural areas. Generally, *Ae. albopictus* has blood-meal preference on mammal, nonetheless this species can also be found has blood-meal preference on human at several areas, depends on its native and invasive ranges. Based on their behavior, *Ae. aegypti* is known as the most anthropophilic mosquito because its habitat and its capability to repeatedly bite human on daily basis. Therefore, it may also have contribution to cause many dengue fever cases in Indonesia [3, 4].

DENV transmission is mediated by DENV-infected female *Aedes* mosquito biting during blood feeding in host [2, 5–7]. The female mosquito is infected by the virus through previous blood feeding in dengue infected host. The viruses from infected host blood travel to the midgut of mosquito and replicate there. Then the viruses enter circulatory system and spread to salivary glands and other tissues. At this stage, the mosquito has been able to transmit the viruses to host during the blood feeding [1, 2, 5, 8]. For the successful blood feeding, mosquito needs to encounter blood coagulation and host's immune response[5, 9]. Hence, mosquito injects saliva containing anti-hemostasis, anti-inflammatory, and immunomodulatory components. Anti-hemostasis components hinder hemostasis such as blood clotting and platelet aggregation evolving vasodilatation to keep the blood flowing during blood feeding [8, 10]. Immunomodulatory components can modulate immune response in host contributing to enhancement of virus transmission in host [11, 12]. Immunomodulatory components in mosquito saliva can alter the Th1 to Th2 immune response. This immune response alteration can suppress the pro-inflammatory cytokines such as IFN-γ and IL-2 therefore escalating the anti-inflammatory cytokines such as IL-10 and IL-4. Suppression of pro-inflammatory cytokines promotes the initial virus infection and supports the virus spread in host. However, this immune response shifting to Th2 can give protection to host combating vector salivary components and preventing the virus infection [11].

The salivary glands of *Ae. aegypti* contain numerous immunogenic components which are capable to induce immune responses in host. Repeated exposure of these immunogenic components can build immunity in host against viral infection, which is transmitted by *Ae. aegypti*. It implies that immunomodulatory components are potential candidates for dengue vaccine development [8, 12]. Previous study identified 56 kDa and 31 kDa immunogenic proteins in the salivary of *Ae. aegypti*, which was specifically detected in people residing in endemic areas [13]. This study result indicates that person residing in endemic areas have antibodies anti-56 kDa and anti-36 kDa proteins because they are frequently exposed by *Ae. aegypti* saliva [14]. However, the host's humoral immune response (IgG) against the 56 kDa immunogenic protein from the salivary glands of *Ae. aegypti* is still unknown. Therefore, this study was conducted to observe host's immune response (IgG) against 56 kDa immunogenic protein through in-vivo study in BALB/c mice (*Mus musculus*).

2 Materials and Methods

2.1 Rearing of Ae. aegypti

In this experiment, we used *Ae. aegypti* which was reared on laboratory scale. Rearing of *Ae. aegypti* was carried out on laboratory scale under controlled conditions at 28 °C with

60% relative humidity at Animal Care Unit, Biology Department, Faculty of Mathematic and Natural Sciences, University of Jember, Indonesia. The rearing was initially started by collecting the larvae from the clean water tank or clean water reservoir around areas of Sumbersari and Arjasa, Jember, Indonesia. These areas were chosen for larvae collection due to the behaviour of people in these areas that put water reservoir or water tank outdoor and let them opened, therefore serving good place for mosquitos to lay their eggs. The larvae that had been collected then were maintained into adult mosquitos. Firstly, the larvae were placed in a container filled with clean water until they were developed into pupae. After that, the pupae were moved into the cage with dimension of 1x1x1 m for maintaining the pupae into adult mosquitos. This dimension of mosquito cage was estimated sufficient to accommodate our research demand which was approximately 50–100 mosquitos in a day for salivary glands isolation. After the adult mosquitos emerged from pupae, the male mosquitos need some times to be sexually mature for mate, however the female mosquitos are ready for mate right after of their emergence [15]. The adult mosquitos were given nutrition with 10% sucrose solution and a Wistar rat caged in a small cage for the blood feeding of female mosquitos. The male and female mosquitos need sugars for their nutrition and only female mosquitos need to do blood feeding to support their egg development [15]. The blood feeding was done twice a day. The blood feeding was carried out by putting the Wistar rat in the mosquito cage for 4 h. After 4 h, the Wistar rat was taken out and was replaced with new Wistar rat to meet the need of blood feeding with total duration of 8 h per day.

2.2 Ae. aegypti Salivary Glands (SG) Isolation

Identification of mosquito species and sexuality was done prior to salivary glands dissection. Identification of *Aedes* species was carried out by observing the line marking on the dorsal (mesonotum). *Ae. aegypti* is characterized by the lines mark like lyre with two curved lines and two straight white lines, whereas *Ae. albopictus* has only single white line [16]. Sexuality of mosquito can be easily identified by the antenna structure. The antenna of male mosquito has bushy and plumose hairs, whereas the female mosquito has smaller and less dense hairs [17]. The salivary glands were collected from female *Ae. aegypti,* 7–10 days old counted from their first blood feeding. The mosquitos were anesthetized prior to salivary glands dissection by placing at 4 °C for 20 s. The salivary glands were dissected using microdissection needle under stereomicroscope. The salivary glands could be easily distinguished from other internal organs by looking at their distinctive lobe shapes as depicted in Fig. 3. The isolated salivary glands were put into a microtube containing 1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF) in Phosphate Buffered Saline (PBS) pH 7.4 then was stored at -20 °C until further use.

2.3 SG Proteins Extraction and Profile Analysis

The collected salivary glands were extracted to derive total protein extract from *Ae. aegypti* SG. The total SG protein extract then was added with Laemmli sample buffer (1:1 (v/v)) and boiled at 95 °C for 3–5 min. SDS-PAGE was done to analyze SG protein extract profile. SDS-PAGE was utilized using stacking gel 4%. And separating gel 12%. Electrophoresis was done for 90 min using constant voltage of 100 V and running

buffer pH 8.3. SDS gel was soaked in Coomassie Brilliant Blue (CBB) staining solution for 60 min, then it was soaked into 3 different destaining solutions alternately for 30 min each to vanish the unbound staining solution to protein bands. Prestained protein marker (Promega, V8491) was used for protein bands size estimation. Protein band with molecular weight of 56 kDa was cut and collected into a microtube containing running buffer pH 8.3 and stored at 4°C for purification step.

2.4 Purification of 56 kDa Protein

Electroelution method was used in the purification step of 56 kDa protein. The collected 56 kDa protein bands were put into cellophane membrane which was previously filled with running buffer pH 8.3. The cellophane membrane was closed tightly and placed horizontally within electrophoresis chamber containing running buffer pH 8.3. Electroelution process was done until the protein bands color was clear. The resided solution in cellophane membrane was transferred into new cellophane membrane and dialyzed using PBS pH 7.4 for 24 h. PBS solution was renewed every 8 h. The solution in cellophane membrane then were precipitated using cold ethanol absolute (1:1 (v/v)) for overnight. Then, the solution was centrifuged at 12000 rpm at 4°C for 15 min and air dried. Proteins as the result of precipitation step were added with Tris-HCl buffer (0.05 M, pH 6.8). Protein concentration was measured using nano drop.

2.5 In-Vivo Assay

In-vivo assay was performed using male BALB/c mice (*Mus musculus*) 2–3 months old with 30 gr of weight. The male BALB/c mice (*Mus musculus*) were obtained from Pharmacy Veterinary Center, Surabaya, Indonesia. In this experiment, we used 24 mice that were divided into three groups, which was each group contained 8 mice. These groups were Group-A which was injected with Tris-HCl buffer (0.05 M, pH 6.8), Group-B which was injected with adjuvant, and Group-C which was injected with 56 kDa protein 0.1 μg/μl from *Aedes aegypti* salivary glands mixed with adjuvant with ratio 1:1 (v/v). The injection was done every two weeks in total six weeks of treatment. In Group-B and Group-C, a Complete Freund's Adjuvant was utilized in initial injection, meanwhile Incomplete Freund's Adjuvant was utilized for subsequent injection. Blood samples of treated animals were collected every two weeks to ensure detectable IgG level. Blood samples were taken from the sinus orbitalis to get sufficient amounts of serum from the animal model. During the blood sample collection, 1 mL blood sample from each individual was collected in a microtube and was stored in ice box to avoid sample damage. The protocol in this in-vivo assay was approved by Ethic Committee of Medical Research Faculty of Dentistry, University of Jember under No.1034/UN25.8/KEPK/DL/2020.

2.6 Blood Serum Preparation

The blood sample which had been collected in a microtube then be processed further to get the serum for IgG analysis. The serum was separated from other blood components by firstly placing the blood sample in microtube at room temperature for 20–30 min

until two layers were formed. The clear layer on the top contains serum and the red layer on the bottom contains other blood components. The clear layer was taken out by micropipette and was put into new microtube and then be centrifuged at 3000 rpm at 27 °C for 15 min. The final volume of serum which was attained from 1 mL of blood sample was ± 350 uL in average. The serum then was put into new microtube and stored at -20 °C until needed.

2.7 IgG Analysis

IgG level in host's serum was quantified by indirect Enzyme-Linked Immunosorbent Assay (ELISA) method. IgG level was measured in individual and population mice sera. Analysis of population IgG level was done using pooled serum of each mice group. We used 56 kDa protein as antigen, serum from treated animals as the primary antibody, and anti-mouse IgG enzyme conjugated as secondary antibody. The first step was coating each well of 96-well microplate using 50 µl antigen diluted in bicarbonate buffer for overnight (12 h). After that, each well was washed using 250 µl Phosphate Buffer Saline Tween (PBST). Then, 200 µl blocking buffer (1% BSA diluted in PBST) was added into each well then incubated for 2 h. Each well was washed several times then added with 50 µl primary antibody in blocking buffer (1:100) and incubated for 1 h. Then, each well was washed using 250 µl PBST and added 50 µl secondary antibody in blocking buffer (1:1000) and incubated for 1 h. For visualization step, each well was added 50 µl TMB substrate at room temperature for 30 min. Then 50 µl H_2SO_4 1 M was added to each well to stop the reaction. The absorbance value was measured with Optical density (OD) 450 nm.

3 Results and Discussion

3.1 Ae. aegypti *Identification*

Ae. aegypti and *Ae. albopictus* are vectors for DENV transmission to humans. *Ae. aegypti* is anthropophilic mosquito, lives in near human habitats, hence *Ae. aegypti* is primary vector that most effective to help DENV transmission. *Ae. albopictus* is the secondary vector of DENV transmission [2]. *Ae. aegypti* and *Ae. albopictus* are morphologically identic, nonetheless these species are easily distinguished from distinct characteristics in their adult form [18].

Ae. aegypti and *Ae. albopictus* can be easily distinguished by their thorax characteristic. *Ae. aegypti* has lines mark like lyre with two curve lines named Lyre Marking (LM) and two straight lines named Submedial Longitudinal Line (MLI) (Fig. 1.A). *Ae. albopictus* only has one straight line on the thorax (Fig. 1.B) [16].

The sexuality of *Ae. aegypti* can be identified easily by observing several parts in their head (caput), those are antenna, proboscis, and maxillary palp. The proboscis and antenna of male mosquito are longer than the female mosquito, the male mosquito has bushy and plumose hairs, whilst the female mosquito has smaller and less dense hairs. The male mosquito maxillary palps seems longer than female, the length of the maxillary palp in male is equal to their proboscis length (Fig. 2) [17].

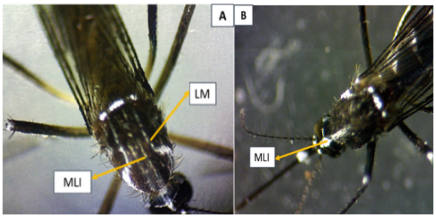

Fig. 1. Thorax of *Ae. aegypti* (A) and *Ae. albopictus* (B); Lyre Marking (LM); Sub-medial Longitudinal Line (MLI) (Olympus stereo microscope magnified 12X, Image: Optilab camera).

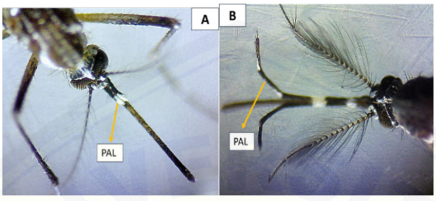

Fig. 2. Maxillary palp (PAL) of *Ae. aegypti*, female (A) and male (B) (Olympus stereo microscope magnified 40X, Image: Optilab camera).

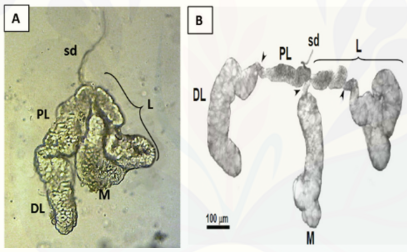

Fig. 3. (A) Female *Ae. aegypti* salivary gland (Olympus stereo microscope magnified 400X, Image: Optilab camera); (B) Female *Ae. aegypti* salivary gland [17], distal lateral (DL), Proximal Lateral (PL), Salivary Duct (sd), lobus single medial (M), lobus lateral (L).

3.2 Ae. aegypti Salivary Glands (SG) Isolation

Ae. aegypti salivary glands consists of one pair salivary glands with six lobes in total. These two parts are connected by salivary duct. Each part consists of three lobes, one part in the middle (single medial) and two parts in the lateral (lateral lobes). The lateral lobe is distinguished into two parts are proximal lateral (PL) and distal lateral (DL) (Fig. 3) [19].

3.3 Purification of 56 kDa Protein from SG Total Protein Extract of Ae. aegypti

The protein profile from *Ae. aegypti* SG was evaluated by SDS-PAGE method. The SDS-PAGE obtained 31 kDa and 56 kDa protein bands (Fig. 4). Both proteins are reported immunogenic and expected can modulate the host's immune response who

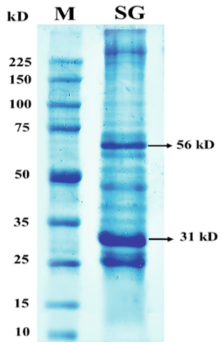

Fig. 4. Protein profile of *Ae. aegypti* salivary glands; Protein Marker (Promega, V8491) (M); *Ae. aegypti* salivary glands extract (SG) (Image: Scanner Canon MP287).

lives in endemic areas [13]. Protein band with molecular weight of 56 kDa was cut and collected for purification step using electroelution method. The purification step was done three times and yielded three different concentrations of 56 kDa protein extract 0.17 mg/ml, 1.59 mg/ml, and 1.99 mg/ml. These 56 kDa protein extracts then were used for in-vivo assay.

3.4 IgG Analysis

In-vivo assay was executed by grouping BALB/c mice into three groups of treatment, they were Group-A which was injected with Tris-Cl 0.05M, Group-B which was injected with adjuvant, and Group-C which was injected with 56 kDa immunogenic protein 0.1 μg/μL + adjuvant. IgG level was analyzed using ELISA method. From the analysis of individual IgG level, it showed that the mice treated with 56 kDa immunogenic protein (Group-C) had gradual increase of IgG level, meanwhile the other groups tend to be steady (Fig. 5). This result is supported by the similar result on IgG analysis in population, it showed that population of Group-C had higher IgG level than other groups after given exposure of 56 kDa protein (Fig. 6). Furthermore, the IgG level from Group-C population was the highest at the end of treatment. According to the analysis results, the repeated exposure of 56 kDa protein in Group-C might contribute to the gradual increase of IgG level in this group, whereas no exposure of 56 kDa protein in Group-A and Group-B might cause the IgG level in these groups tend to be steady until the end of treatment.

Based on the analysis results, it confirms that 56 kDa protein from *Ae. aegypti* SG is immunogenic, considering its ability to elevate IgG production in mice group C. IgG can be used as biomarker for immunogenic proteins existence because IgG is only produced when the B cell is activated by specific immunogens [20]. Activation of B cell induces B cell proliferation and differentiation into plasma cell and memory cell. Plasma cell is responsible for antibodies production, include IgG [21]. Specific antibodies production against mosquito salivary proteins is closely related to the level saliva proteins exposure [22, 23]. The increase of IgG level in mice serum reflects positive correlation to the increase of 56 kDa immunogenic protein exposure in mice. The steady IgG level in mice

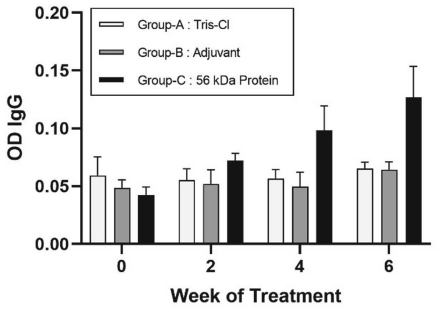

Fig. 5. Humoral immune response (IgG) of individual BALB/c mice (*Mus musculus*).

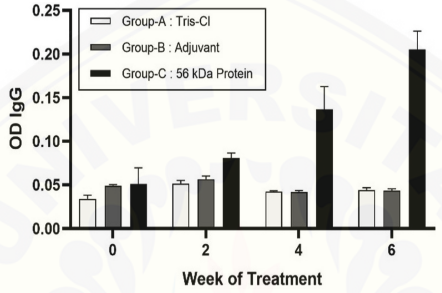

Fig. 6. Humoral immune response (IgG) of population BALB/c mice (*Mus musculus*).

A and Group-B serum indicates the absent of 56 kDa immunogenic protein exposure in these mice groups [22, 24, 25].

Immunogenic components in mosquito saliva can alter normal anti-viral immune response Th1 to Th2 [26, 27]. The polarization of immune response to Th2 inhibits the Th1 cytokines secretion such as IFN-γ and IL-2 but augments Th2 cytokines secretion such as IL-4 and IL-10 [10, 26, 27]. IL-10 and IL-4 are associated with B cell proliferation and differentiation into plasma cell and memory B cell. IL-10 and IL-4 promote B cell differentiation into plasma cell secreting IgG [28]. Memory B cell contributes to the rapid response toward secondary antigen exposure, therefore inducing faster B cell proliferation and differentiation into plasma cell affecting greater antibodies production [29, 30]. Based on these explanations, the elevated IgG level in mice after given repeated exposure of 56 kDa protein from *Ae. aegypti* SG is the result of Th2 immune response modulation. Consequently, it drove the increase of IgG production along with the increase of 56 kDa protein level exposure [31, 32].

This study results suggested that the higher exposure level of 56 kDa immunogenic protein from *Ae. aegypti* SG would increase the humoral immune response (IgG) in host. This result is similar to other studies that implied the increase of IgG level in host had positive correlation with the increase of protein exposure from *Ae. aegypti* saliva [24, 33, 34]. Enhancement of IgG level in treated 56 kDa protein mice implies that exposure of 56 kDa proteins from *Ae. aegypti* SG potentially gives protection effects in host against saliva components to prevent dengue virus infection [32]. Furthermore, repeated

exposure of 56 kDa protein will enhance the B cell memory production, therefore it may provide long term humoral immunity in host [28]. In conclusion, this study found that the mice treated with 56 kDa immunogenic protein showed higher IgG level than non-56 kDa immunogenic protein treated mice. IgG level of mice treated with 56 kDa immunogenic protein gradually increased along with the increase of 56 kDa immunogenic protein exposure. These results showed that repeated exposure of 56 kDa immunogenic protein from *Ae. aegypti* SG elevated host's humoral immune response (IgG).

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Authors' Contributions. RO and KS conceived the original idea, designed the analysis, and supervised the study. SW supervised the study. A collected the data, performed analysis, and wrote the manuscript. All authors read and approved the final manuscript.

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