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In vitro and in vivo analysis of IgG antibody response against 47 kDa immunogenic protein from salivary gland of *Aedes albopictus*

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ABSTRACT. Dengue Hemorrhagic Fever (DHF) is a disease caused by the dengue virus. The virus is transmitted to humans through the blood-feeding activity of *Aedes albopictus*. This transmission is facilitated by specific proteins of salivary glands mosquito. Based on the previous studies, there are three immunogenic proteins were detected from salivary glands of *Ae. albopictus*, i.e 31, 47, and 67 kDa. The 47 kDa protein is one of the proteins suspected to be a serpin protein. These proteins are capable of inducing an immune response in the host. This study aims to assess the capacity of the 47 kDa immunogenic protein derived from the salivary glands of *Ae. albopictus* to elicit host immune responses. This is achieved through IgG analysis conducted both in vitro and in vivo analysis. In vitro IgG antibody response analysis was performed on human serum samples from healthy people, DHF patients and neonates collected from Jember. In vivo IgG antibody response analysis was performed on mice (*Mus musculus*) that had been injected with 47 kDa protein. IgG level measurement using the ELISA indirect method. According to in vitro examination of IgG antibody response in human samples against 47 kDa salivary protein, the highest IgG levels were detected in healthy samples followed by dengue patients and neonates. This suggests that the 47 kDa protein from *Ae. albopictus* salivary glands is recognized by human serum and developed into a biomarker for mosquito bites. In vivo examination of the IgG antibody response in mice (*M. musculus*) injected by 47 kDa protein revealed that the IgG antibody might be increased by repeated exposure to 47 kDa protein. The highest IgG level was detected in the 6th week after repeated exposure. Repeated exposure of the 47 kDa salivary protein from *Ae. albopictus* have been demonstrated to elicit a humoral immune response in mice.

Keywords: 47 kDa immunogenic protein; *Ae. Albopictus*; IgG antibody; salivary gland

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INTRODUCTION

Dengue Hemorrhagic Fever (DHF) is one of the endemic diseases that exist throughout the tropics and parts of the subtropics. Dengue fever is caused by *Dengue virus* (DENV) of the genus *Flavivirus* family *Flaviviridae* (Nonyong *et al*., 2021). There are four serotypes virus i.e DENV-1, DENV-2, DENV-3, DENV-4 (Wang *et al*., 2020). DENV is transmitted through mosquito bites *Aedes aegypti as* the primary vector and *Aedes albopictus* which is also a potential primary vector (Ahebwa *et al*, 2023). *Ae. albopictus* considered one of the most invasive species globally because of its strong ability to adapt to new environments (Dalpadado *et al*., 2022). Due to its adaptability *Ae. albopictus has* been confirmed as a potential primary vector in some areas where it is rare or not *Ae. aegypti* (Erickson *et al*., 2010). Dengue virus transmission occurs when female mosquitoes blood feed on infected humans. Once inside the mosquito, the virus replicates in the midgut before spreading to other parts of the body, including the salivary glands (Islam *et al*., 2021).

Vector saliva contains several substances that function as antihemostatic agents, anti-inflammatory and immunomodulators [e.g., by suppressing host dendritic cell maturation or altering cytokine profiles). These substances function to facilitate the blood feeding process in the host and can determine the success of pathogen transmission in the host. The vasodilator components in saliva help mosquitoes in the process of blood-feeding. *Aedes* mosquito saliva contains immunomodulatory components that can influence the host's immune response, potentially enhancing viral transmission

and affecting disease (Guerrero *et al*., 2020). The immunomodulatory protein can influence the host's adaptive immune system. These proteins have been shown to modulate immune responses, potentially affecting the production of immunoglobulins such as IgG. Additionally, research indicates that mosquito salivary proteins can elicit specific IgG responses in humans, suggesting their role in modulating the adaptive immune system (Oseno *et al*., 2022).

Among the immunomodulatory salivary proteins, proteins from *Ae. albopictus*, particularly the 31, 47, and 67 kDa proteins, play a role in inducing the host immune response (Oktarianti *et al*., 2021a). These protein is one of the proteins suspected to be a serpin protein. Serpin inhibits the activity of serine proteases involved in the coagulation process (e.g. thrombin), thereby preventing blood clotting at the bite site (Meekins *et al*., 2017). Serpins can also suppress the host's local immune response, by disrupting proteolytic cascades (e.g., complement activation or inflammation) (Bao *et al*., 2018). Additionally, serpin proteins are capable of inducing an immune response in the host (Gulley *et al*., 2013). Because serpin's potential to stimulate immune responses, it is necessary to conduct research to determine the ability of the 47 kDa protein from the salivary glands of *Ae. albopictus* to induce a host immune response by conducting IgG analysis both in *vitro* and *in vivo*. Thus, this study can provide a deeper understanding of the mechanisms of interaction between vector and host, as well as open opportunities for the development of diagnostic and therapeutic strategies based on immune responses to serpin proteins, which ultimately may contribute to the control of mosquito-borne diseases.

MATERIALS AND METHODS

Rearing and identification species. Mosquitoes collection was carried out by collecting the larvae, then continued by the rearing process. Larvae were reared in a container temperatur 26–28 ºC, relative humidity ~70-80%. Identification of *Ae. albopictus* based on morphological characteristics of the mesonotum, mesopimeron and anterior mid femur. The mesonotum of *Ae. albopictus* mosquitoes has thick white median-longitudinal lines on the dorsal side of the thorax. The mesopimeron of *Ae. albopictus* mosquitoes has two white scales that form a V-shape on the lateral thorax. The anterior mid femur of *Ae. albopictus* mosquitoes does not have white longitudinal lines and is only completely dark black without white scales (Rueda, 2004).

Salivary gland protein isolation and extraction ***Ae. albopictus*****.** Salivary gland isolation using the microdissection method (Schimd *et al,* 2017). Mosquitoes will be isolated under a stereo microscope with a drop of 0.5% NaCl on a glass object. NaCl functions as an isotonic solution to the salivary gland cells thereby preventing the lysis of the salivary glands. The salivary glands obtained were then put in microtube sterile containing 10 µL of 1 mM PMSF in PBS pH 7.4. The volume of PMSF solution in PBS is 1:1 proportional to the number of salivary glands stored in its microtube at a temperature of -20 ºC. The salivary glands obtained were then added with a 10 µL loading *buffer* on the sample and heated using a thermoshaker at a temperature of 95 ºC for 4 minutes.

Protein fraction isolation 47 kDa. Isolation of the 47 kDa protein fraction using the SDS-PAGE method (*Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis*). SDS-PAGE was performed with a 12% separating gel and a 4% stacking gel and was carried out at a voltage of 150 V (constant volts) for 60 minutes in a buffer electrode of pH 8.3 (Wathon *et al*., 2023). The protein sample loaded into each gel well was 20 ul. Gel staining was carried out in a staining solution with Coomassie Brilliant Blue (CBB) R250 dye and continued with the destaining process. The 47 kDa target protein was then cut aseptically and stored in buffer electrode pH 8.3 at 4 ºC.

Protein fraction purification 47 kDa. Purification was carried out using electroelution and dialysis methods. The electroelution was carried out at a constant voltage of 120 V for 1 hour at a temperature of 10 ºC until the gel color fades. The liquid in the membrane is transferred to the new cellophane membrane. Then dialysis for 12 hours in 500 mL of PBS pH 7.4 temperature 4 ºC (Wathon *et al*, 2023).

In vitro analysis of human immune response (IgG) against 47 kDa protein. In vitro alalysis of human immune response (IgG) against 47 kDa protein from the salivary glands (SG) of *Ae. albopictus* was determined using ELISA indirect (Oktarianti *et al*., 2021b). The indirect ELISA where primary and secondary antibodies are used, the primary antibody is the sample serum and the secondary antibody is anti-human IgG. Procedure *indirect* ELISA begins with coating antigen *well microplate 96 well.* Then it was washed with 250 µL PBST (*Phosphate Buffer Saline Tween*). Once it's done blocking *buffer* in each well by adding 200 µL. Well then washed again with PBST and added primary antibody. Then, secondary antibody was added and TMB substrate was added. Substrate was added in a dark room for 30 minutes at room temperature until the color changed. Then 1 M sulfuric acid was added. ELISA has a working principle of bond interaction between antigen and antibody. The color density of the ELISA results was measured using ELISA *reader* with an OD of 450 nm

In vivo analisis of mice immune response (IgG) against 47 kDa protein. The animal model used was ddY strain mice, which originated from Pharmacy Veterinary Center, Surabaya, Indonesia. The mice used were male and 3–4 months old. The number of mice samples used was 21 mice which were grouped into 3 treatment groups, with each group consisting of 7 mice. The group division was as follows: Group (A) negative control injected with PBS pH 7.4; Group (B) adjuvant control injected with adjuvant; and Group (C) treatment group injected with 47 kDa protein (0.1 µg/µL) mixed with adjuvant in a ratio of 1:1. The first injection in the treatment group used a 47 kDa protein extract mixed with CFA (Complete Freund's Adjuvant). Subsequent injections used IFA (Incomplete Freund's Adjuvant). The injections were performed subcutaneously on the backs of the mice. Injections were carried out every 2 weeks for six weeks. In vivo analysis IgG of mice immune response (IgG) against 47 kDa protein from the salivary glands (SG) of *Ae. albopictus* was determined using ELISA indirect. The procedure for measuring IgG in mouse serum is identical to the procedure used for measuring IgG in human serum, except that the secondary antibody used is anti-mouse IgG.

Blood serum preparation. Human sera samples were taken from healthy people (15-40 years old), infant (neonatus) and DHF patients living in Jember, East Java, a region within the endemic range for Dengue. All participants gave written, informed consent to take part in the study. The collecting protocol was approved by the Ethical Committee of the Faculty of Medicine, Jember University number 2068/UN25.8/KEPK/DL/2023. Mice blood samples were taken from the eye to be exact orbital *sinus.* Blood sampling was carried out before and after injection in the 2nd, 4th, and 6th weeks.

Data analysis. The absorbance value was then analyzed quantitatively by test *one-way* ANOVA using SPSS 16. If the ANOVA results show a significant difference, then proceed with the test *Duncan's Multiple Range Test* (DMRT) to find out which treatment groups have significant differences.

RESULTS AND DISCUSSION

To ensure that the salivary proteins used in this study from *Ae. albopictus*, species identification was performed on the adult mosquitoes. Mosquito *Ae. albopictus* can be identified by looking at several parts of its body, i.e mesonotum on the dorsal thorax, on the mesepimeron on the lateral side of the thorax, and on the anterior mid-femur (Rueda, 2004). Mosquito mesonotum *Ae. albopictus* have lines median-longitudinal thick white on the dorsal side of the thorax. Mesopimerone in mosquitoes *Ae. albopictus* has two white scales that form like the letter V on the lateral thorax. Anterior section mid *femur* mosquito *Ae. albopictus* does not have longitudinal white lines and is only dark black all over without any white scales (Supriyono *et al*., 2023). Mosquito morphology *Ae. albopictus* can be seen in Fig. 1.

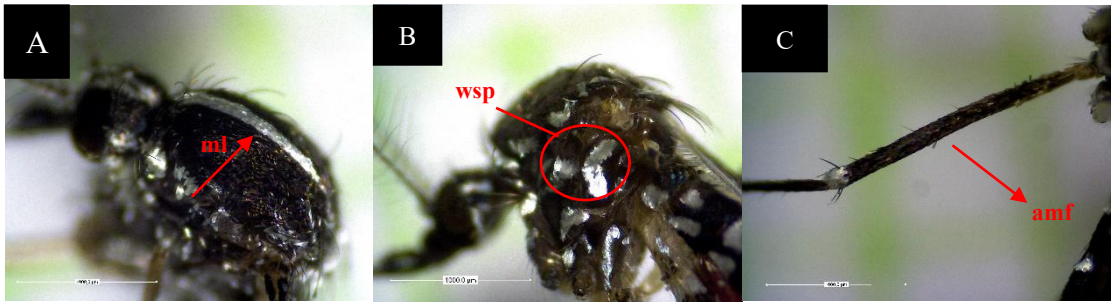

Fig. 1. Morphology *Ae. albopictus* (Olympus Stereo NIKON SMZ745 Microscope, 50x magnification); (A) Mesonotum; (B) Mesepimeron; (C) Anterior mid- femur (amf); median longitudinal (ml); white scale patches (wsp)

Differences in mosquito morphology *Ae. albopictus* male and female can be seen in Fig. 2. Antenna on *Ae. albopictus* bushy male (*pulmose*) but females have antennae with sparse hair (hairy) (Supriyono *et al*., 2023). Another difference that can be observed in female *Ae. albopictus* mosquitoes is that they have a longer proboscis than the palpus, while males have a proboscis the same size as the palpus (Biswas & Banerjee, 2016).

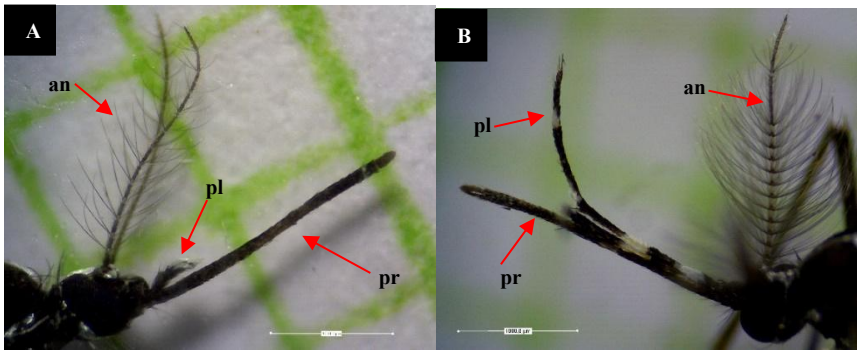

Fig. 2. Morphological differences *Ae. albopictus* male and female (Olympus Stereo NIKON SMZ745 Microscope, magnification 40x); (A) *Ae. albopictus* male; (B) *Ae. albopictus* female; Palpus (pl); Proboscis (pr); antenna (an)

The structure of the salivary glands of *Ae. albopictus* is a pair, connected by the salivary duct (SD) as shown in the Fig. 3. Each salivary gland consists of three lobes, i.e two lateral lobes (L) and one median lobe (M).

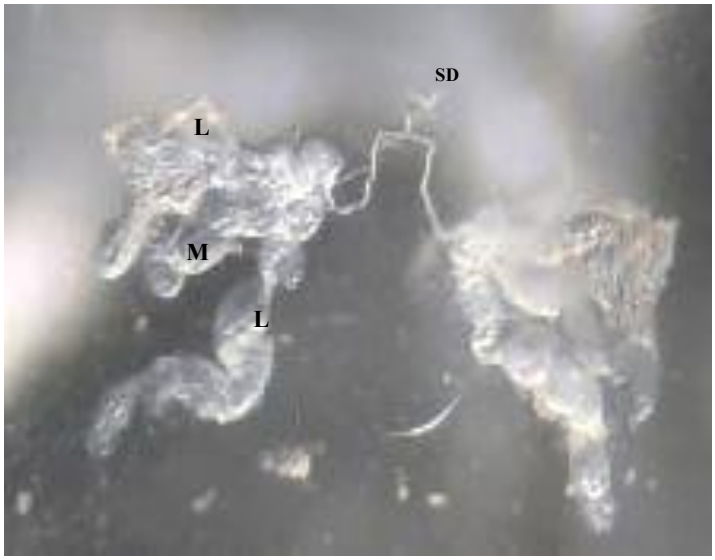

Fig. 3. Salivary glands of female *Ae. albopictus* (Olympus NIKON SMZ745 Stereo Microscope, 50x magnification)

Protein separation was carried out using SDS-PAGE to produce a 47 kDa band. Fig. 4 shows the protein profile of the salivary glands of *Ae. albopictus*. In order to get pure protein, the 47 kDa protein was subsequently separated from the electrophoresis gel for further purification.

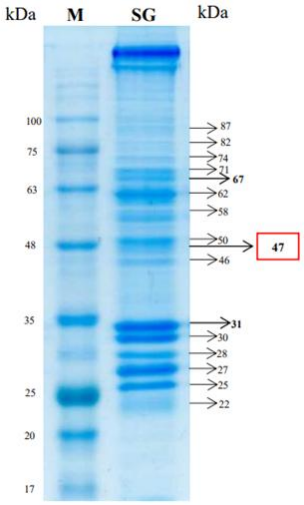

Fig 4. Salivary gland protein profile *Ae. albopictus* (EPSON L3210 scanner); salivary gland extract *Ae. Albopictus* (SG); Blue Elf Prestained Protein Marker (M)

In vitro analysis of human immune response (IgG) against 47 kDa immunogenic protein. In vitro analysis of human humoral immune response (IgG) in this study was carried out using the ELISA (Enzyme-Linked Immunosorbent Assay) indirect method. An indirect ELISA that uses enzymatic reactions to identify primary antibodies was used to detect IgG antibodies (Pineda *et al*., 2016; Yin *et al*., 2015). The results of human immune response (IgG) from individual sera sample of neonate, healthy people and DHF patient can be seen at Fig. 5 and population sample (pool serum) at Fig. 6.

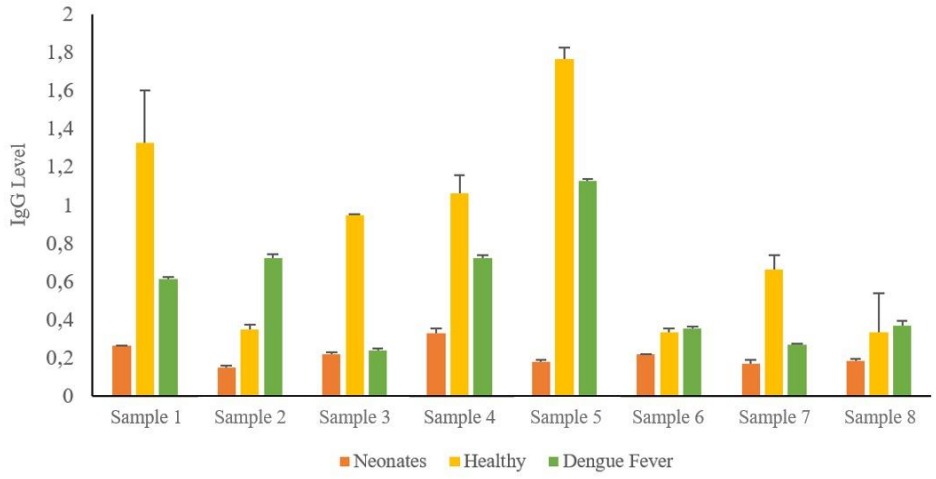

Fig. 5. Individual IgG level of samples in neonates, healthy people, and DHF patients from Jember endemic area

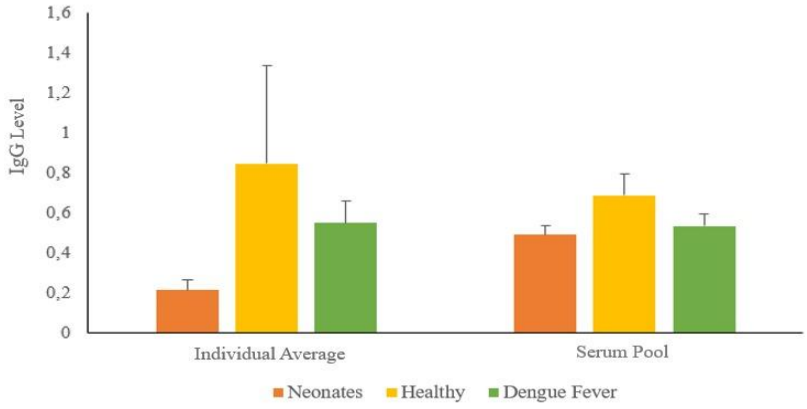

Fig. 6. The IgG level average individual sample and IgG level population (serum pool) of neonates, healthy people and DHF patients

The IgG level from individual neonate, endemic healthy people, and DHF patients ranged from 0.149-0.327; 0.333-1.764; 0.238-1.123. The IgG antibody response showed a bond between the 47 kDa protein and IgG antibodies in human serum, this indicate that the 47 kDa protein has immunogenic. Protein fractions that have immunogenic properties can induce the adaptive immune responses (Dembic, 2015). Based on the data shown in the graph, it can be seen that there were variations in individual immune responses. The immune response individual of dengue patient and healthy people were varies, it is caused grade of exposure to each individual were different, therefore each individual has different response (Franceschi *et al*, 2017). Neonate samples was detected has IgG low levels, compare to healthy people and DHF patient. The low IgG antibody in the neonate was originated from the mother through the placenta during pregnancy. IgG is the only antibody from the mother can penetrate to baby through the placenta (Calvert & Jones, 2017; Mayer & Joseph, 2013; Oktarianti *et al*, 2021b). DHF patients exhibit lower serum IgG levels compared to healthy individuals and controls. This reduction occurs because pre-existing dengue-specific IgG binds to viral antigens, forming immune complexes that are rapidly cleared from the circulation by macrophages and the complement system. As a result, the majority of IgG becomes bound and eliminated, leading to an apparent decrease in free circulating IgG levels (Wang *et al*, 2006).

Fig. 6 showed IgG immune response of population samples were similar to individual samples, the highest IgG antibody response was found in healthy person followed by DHF patient and finally the neonate samples, respectively. Antibody in the body are influenced by the intensity of exposure to the salivary glands of *Ae. albopictus* (Oktarianti *et al*., 2021b). In endemic area, both healthy people and DHF patients are more likely to develop antibodies against salivary exposure to *Aedes* (Doucoure *et al*., 2013; Oktarianti *et al*, 2021b). An area's mosquito density is correlated with the human immunological response to IgG (Billingsley *et al*., 2006; Oktarianti *et al*, 2021b). The analysis's findings show that endemic individuals have greater IgG levels than the neonate. One of the host's defense mechanisms against the saliva vector antigen is the IgG reaction. After the host has been repeatedly exposed by the mosquito vector during the blood-feeding, this mechanism will take place. The results of the investigation show that endemic individuals have higher IgG level than neonates because the healthy people in the endemic area were also exposed more than once. After repeated exposure to arthropod saliva, the host immune responses will alter adaptive immune responses, resulting in Th 2. B cells will be activated, becoming plasma cells that will produce specific antibodies such as IgG (Doucoure *et al*., 2013; Fontaine *et al*., 2011; Oktarianti *et al*, 2025).

In vivo analysis of mice (*Mus musculus*) immune response (IgG) against 47 kDa immunogenic protein. The IgG immune response in mice population of group A (negative control), group B (adjuvant control), and group C (treatment 47 kDa + adjuvant) can be seen in Fig. 7.

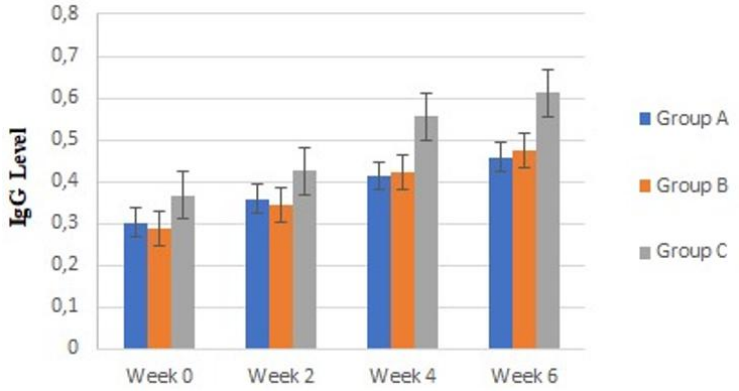

Fig. 7. The IgG level in mice population of group A (control PBS pH 7.4), group B (adjuvant control), and group C (treatment 47 kDa protein + adjuvant)

In general, IgG level in group A and group B from week 0 to 6 tended similar, whereas in treatment group C, IgG levels tended to rise from week 0 to 6. Figure 6 showed the IgG levels in treatment group C was higher than control groups A and B and the highest IgG levels were detected in week 6. The results of the One Way Anova analysis of IgG levels at week 0 with a significance value of $p = 0.108 > 0.05$ showed no significant difference in groups A, B as controls and group C (treatment group). Meanwhile, the results of the One Way Anova test at week 2, week 4 and week 6 showed a significance difference<0.05. Further analysis using the Duncan Multiple Range Test (DMRT) can be seen in Table 1. According Table 1 showed at week 0 there was no significant difference IgG levels in all groups treatment, in contrast at weeks 2, 4 and 6, group C was significantly different from groups A and B. Antibody (IgG) production reaches its peak at week 6, corresponding to the advanced phase of the immune response (typically beginning around week 4). During this stage, long-lived plasma cells are established in the bone marrow, where they continuously secrete high levels of IgG, leading to sustained and elevated antibody titers (Pulendran & Ahmed, 2011).

In group B (adjuvant control) showed IgG increase, because adjuvants can stimulate the non-specific immune system and/or enhance B-cell activation through several mechanisms (APC and cytokine activation, direct B-cell stimulation via TLR). Consequently, even without a clear target antigen, antibody production (including IgG) increases (Awate *et al*., 2013). The results of this study proved that the 47 kDa protein from the salivary glands *Ae. albopictus is* immunogenic because it can increase the humoral immune response (IgG) in experimental mice. This was indicated by an increase in IgG levels with the highest concentration in the 6th week after injection. The immunogenic protein repeated exposure able to activates the adaptive immune system of the host by inducing B lymphocytes production of B cells, which then produce specific antibodies (Oktarianti *et al*., 2025; Doucoure *et al*., 2013). Production of IgG antibodies in the host is used as indicated that there is an immunogenic protein in the vector saliva.

Table 1. The average IgG level of mice (*Mus musculus*) before and after treatment

Treatment	IgG level ($\underline{X}$ ± SD)			
	Week 0	**2nd week**	**4th week**	**6th week**
Group A	0,302±0,037[a]	0,359±0,053[a]	0,414±0,035[a]	0,459±0,020[a]
Group B	0,289±0,056[a]	0,343±0,039[a]	0,422±0,103[a]	0,474±0,104[a]
Group C	0,367±0,099[a]	0,440±0,091[b]	0,556±0,134[b]	0,612±0,159[b]

Numbers in the same column followed by the same letter show no significant difference based on the DMRT test α=0.05. Group A (injected with PBS pH 7.4); Group B (injected adjuvant); Group C (injected 47 kDa protein (0.1 µg/µL) + adjuvant)

The immunogenic proteins signal B lymphocytes to differentiate into plasma B cells and memory B cells (Fontaine *et al*., 2011; Oktarianti *et al*, 2025). Plasma B cells, are the terminally differentiated form of B cells responsible for producing antibodies, thereby playing a crucial role in the humoral immune response. Upon activation by their specific antigen, B cells differentiate into plasma cells that secrete antibodies, predominantly immunoglobulin G (IgG), to neutralize pathogens (Tsai *et al*., 2019). Memory B cells play a role in providing a faster response when the host is exposed to the same antigen (Kitamura, 2021). Thus, secondary exposure will cause higher IgG production.

CONCLUSION

According to in vitro analysis IgG antibody response in human sample to 47 kDa protein of salivary gland of *Ae. albopictus* show that healthy people have the highest IgG level over DHF patient as well as a neonate. Neonate samples was detected has IgG low levels, compare to healthy people and DHF patient. This suggests that the 47 kDa protein from *Ae. albopictus* salivary glands is recognized by human serum and developed into a biomarker for mosquito bites. In vivo examination of the IgG antibody response in mice (*Mus musculus*) injected by 47 kDa protein revealed that the IgG antibody might be increased by repeated exposure to 47 kDa protein. The highest IgG level was detected in the 6th week after repeated exposure.

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