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In vitro analysis of human immune response (IgG) against salivary gland extract of dengue vector from dengue hemorrhagic fever (DHF) endemic area in Jember, Indonesia

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Abstract. The mosquito species *Ae. aegypti* and *Ae. albopictus* are two potential vectors of dengue fever. The salivary glands of these species contain substances that play a role in the transmission of pathogens. These include vasodilators and immunomodulatory compounds. Immunomodulatory components can modulate the host immune system by producing specific antibodies (IgG). This study aims to investigate the human immune response (IgG) against the salivary gland extract of *Ae. aegypti* and *Ae. albopictus*. Samples were collected from individuals who were Dengue patients, as well as healthy individuals and neonates from the Jember endemic area. Results show that the levels of IgG response vary across the individual. Generally, Dengue patients and healthy people in the DHF-endemic area had higher levels of IgG. The highest immune response was found in DHF patients, followed by healthy persons, and finally the neonate samples, respectively.

1. Introduction

Dengue Hemorrhagic Fever (DHF) is a significant health problem in many tropical countries, and is endemic to more than 100 countries, particularly those of Southeast Asia and the Western Pacific [1]. The mortality rate of DHF infection is high, resulting in as many as 1.5 million deaths per year, and causes substantial economic losses as well [2;3]. DHF is caused by the dengue virus (DENV), of which there are 4 serotypes: DENV1, DENV2, DENV3, and DEN4 [4;5]. DENV is transmitted by several species of *Aedes* mosquitoes. In particular, *Aedes aegypti* is known to be a primary vector and *Aedes albopictus* is a secondary vector [6;7]. Dengue virus is transmitted when a mosquito takes a blood meal from an infected person and then a second from another healthy person, thus transferring the virus to the second person [8;9]. Mosquitoes require bloodmeals from vertebrate hosts for adult nutrition and egg development [10].

The success of dengue virus transmission from mosquitoes to humans is caused by immunomodulatory factors and vasodilators in the salivary glands of mosquitoes [11,12]. The immunomodulatory molecules are immunogenic molecules that have immunosuppressive activity on immune response [11;13]. These factors can increase the transmission of pathogens into the host by modulating the host immune response [11]. In addition, immunomodulatory factors can also cause hosts to be more sensitive to vasodilators to facilitate blood feeding [14]. The vasodilator, such as anti-hemostasis, is very important in the blood feeding process. The substance of anti-hemostasis is



inhibiting blood clotting. In addition, the substance of the vasodilator causes dilation of blood vessels through capillary vasodilation [11].

Salivary glands proteins from *Aedes* are known to cause modulation of the host immune response [13]. The modulation occurs due to a shift in the subset of Th1 to subset Th2, which induces B cells to produce specific antibodies (IgG). IgG levels are the gauge used to assess the human immune response to salivary *Aedes* exposure. Research has shown that the saliva of mosquitoes contains immunogenic components [14;15;16]. The saliva induces an IgG-producing response in individuals living in endemic areas and in travelers transiently exposed to vectors in tropical areas [16;17;18]. The development of a natural antibody response in people living in an endemic area (African) results from frequent exposure to mosquito saliva [19]. It has also been reported that IgG levels are significantly higher in persons living in sites with higher *Ae. aegypti* densities [20]. This indicates that vector bites have a positive effect on the host immune response.

There is a protective mechanism in the host immune system which arises from repeated exposure to mosquito vector saliva [21]. Repeated exposure activates the adaptive immune system of the host by inducing B lymphocytes production of B cells, which then produce specific antibodies. During the blood-feeding process the host will produce IgG as a response to the saliva proteins of the *Aedes* mosquitos [20]. This research was conducted to analyze the human immune response (IgG) to salivary gland extracts of *Aedes* mosquitos in the DHF endemic area in Jember, East Java, Indonesia.

2. Material and Methods

2.1. *Aedes* Rearing and Species Identification by Morphological Characteristics

Mosquito larvae were collected and reared under carefully maintained conditions at the Animal Care Unit of the Zoology Laboratory of the Biology Department at the University of Jember. These conditions included a constant temperature of 28°C with 60% relative humidity. Species identification of *Ae. aegypti* and *Ae. albopictus* was done using morphological characteristics of the mesonotum, mesepimeron, and femur. The mesonotum portion of *Ae. aegypti* has a row of white hairs forming two curved white lines, while *Ae. albopictus* has only one white line. Secondly, the mesepimeron of the two species is quite different. *Ae. aegypti* has a collection of white scales that form two separate white patches, while *Ae. albopictus* merges into one. *Ae. aegypti* has a row of white hairs that form a white stripe pattern on the anterior femur, while *Ae. albopictus* does not have this pattern on the femur [22].

2.2. Salivary Gland (SG) Dissection and Protein Extraction

The salivary glands from adult mosquito females were dissected using a fine entomological needle under a stereomicroscope. Salivary glands in PMSF and PBS were added to a concentration containing a lysis buffer (1:1) (Sigma-Aldrich, USA). This buffer was composed of 1.5 mM MgCl₂, 10 mM TrisHCl, 10 mM NaCl, 1% Nonidet P-40, and 2 mM EDTA NaOH (Sigma-Aldrich, USA) [23]. Mixtures were homogenized using a micropistile and then underwent a sonicator water bath (Biosan, Latvia) for 30' and centrifuged at 12.600 rpm 15' 4°C (Eppendorf, Germany). The supernatant was concentrated using epi membrane (10 kDa MWCO) (Corning, USA) and centrifuged again at 10.000 rpm, 4°C. This was repeated several times to create a dense protein concentration from the salivary gland extracts (4 ug/ul) (Invitrogen, USA). Salivary gland proteins were then stored at -20°C until used.

2.3. Blood Sera Collection

Sera samples were taken from healthy people (15-40 years old), infants (neonates) 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 No 148/UN25.8/KEPK/DL/2018 of the Medical Faculty of the University of Jember-Indonesia.

2.4. *In vitro* Analysis of Human Immune Response against Protein Extract of Salivary Gland by ELISA

The human immune response against proteins extracted from the salivary glands (SG) of *Ae. aegypti* and *Ae. albopictus* were determined using ELISA. The 96-well ELISA plates (Corning, USA) were coated with 4 µg/ml (50 µL/well) extracts from the salivary glands of *Ae. Aegypti* and *Ae. albopictus* SG and several wells without SG were added with PBS (Merck, USA) as a negative control. SG were diluted in 0,1 M bicarbonate buffer (pH 9,6) overnight at 4°C. Plates were blocked for 2 hours at 37°C with 1% BSA in PBS-T (PBS, pH 7,4; 0,05% Tween-20S) (Merck, USA). Sera, diluted to 1:100, was added and the result was incubated at 37°C for 1 hour. Washes were done with PBS-T (Merck, USA) between incubations. Fifty microliters of horseradish peroxidase (HRP) and conjugated Rabbit anti-Human IgG (1:5000) (Rockland, USA) were incubated for 1 hour at 37°C. Enzyme activity was determined through incubating for 10 minutes at room temperature with 50 µL tetramethylbenzidine (TMB) (Rockland, USA). Enzymatic reactions were stopped using 50 µL 1M H₂SO₄ (Sigma-Aldrich, USA) for 10 minutes at room temperature. The optical density (OD) of 450 nm was determined with a microplate reader (Bio-Rad, USA).

3. Results and Discussion

3.1. Species Identification and Salivary Gland Structure

Species identification based on morphological characters revealed differences between the mesonotum, mesepimeron, and anterior femur of *Ae. aegypti* and *Ae. albopictus*. *Ae. aegypti* has a row of white hairs which form a white stripe pattern that is a pair of straight white lines and a pair of curved lines like the lyre in the mesonotum, whereas *Ae. albopictus* has only one straight white line (Figure 1). The mesepimeron of *Ae. aegypti* has white scales which form two separate white patches, while on *Ae. albopictus*, these scales are merged into one patch (Figure 2). The femur of *Ae. aegypti* has a row of white hair that forms a straight white line on the anterior part, whereas *Ae. albopictus* only has black hair on the anterior femur (Figure 3).

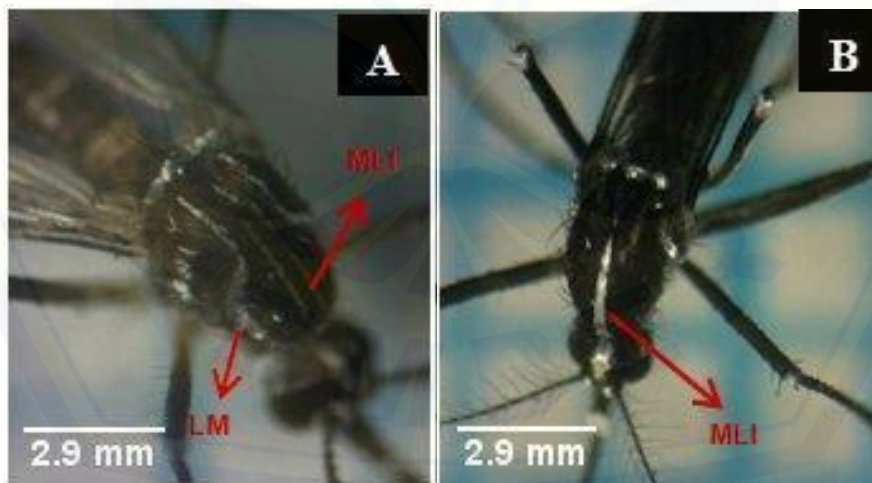


Figure 1. Mesonotum of *Ae. aegypti* (A) and *Ae. albopictus* (B), LM (*Lyre Marking*), MLI (*Submedian Longitudinal Line*) (taken by Stereo Microscope 40x zoom in, camera: OptiLab, Olympus, USA).

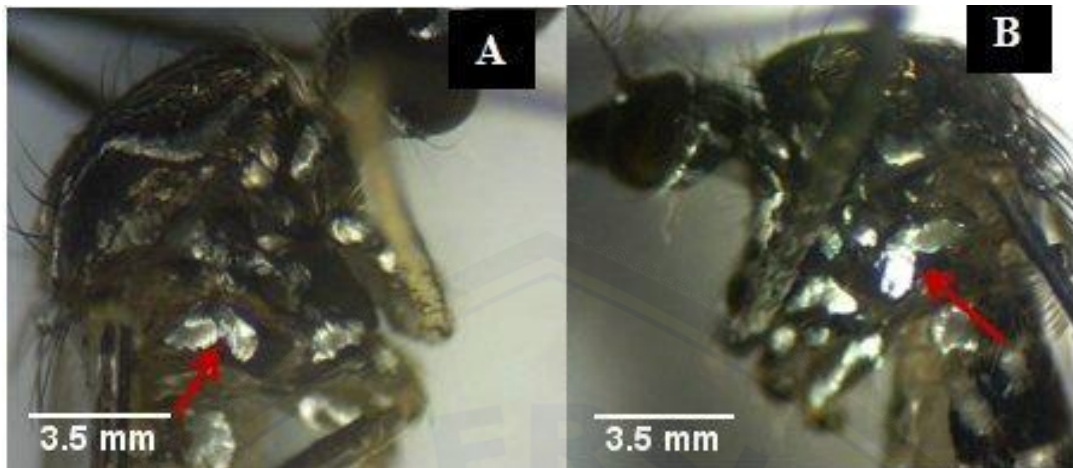


Figure 2. Mesepimeron of *Ae. aegypti* (A) and *Ae. albopictus* (B) (taken by Stereo Microscope 40x zoom in, camera: OptiLab, Olympus, USA).

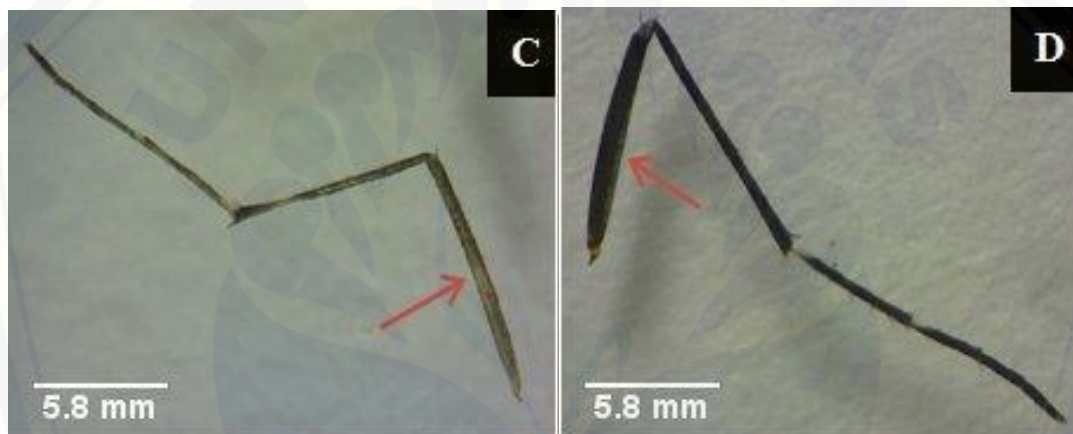


Figure 3. Anterior femur of *Ae. aegypti*(C) and *Ae. albopictus*(D) (taken by Stereo Microscope 40x zoom in, camera: OptiLab, Olympus, USA).

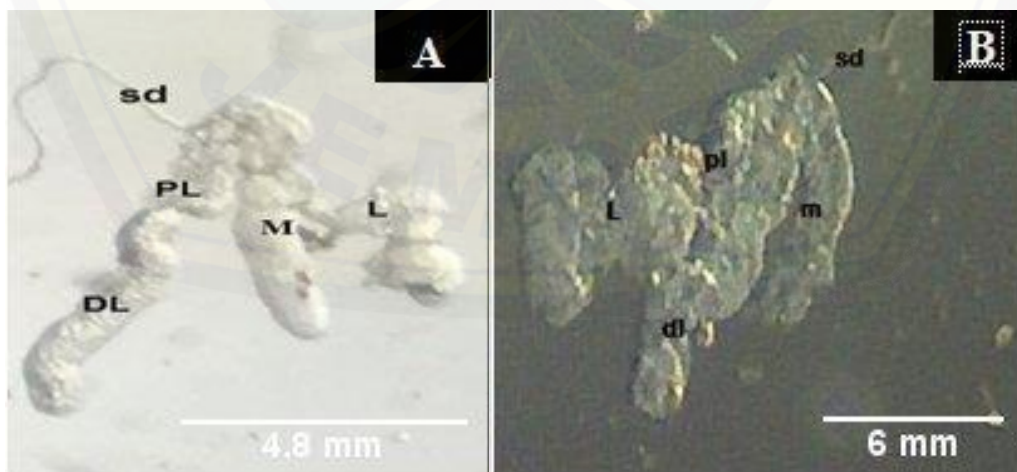


Figure 4. Salivary gland dissected from a female *Ae. albopictus*(A) and *Ae. aegypti*(B), Salivary Duct (SD), Lateral lobes (L), Proximal- Lateral (PL), Distal-Lateral (DL), Medial lobes(M) (taken by Stereo Microscope 40x zoom in, camera: OptiLab (Olympus, USA).

The structures of the salivary gland of adult female *Ae. aegypti* and *Ae. albopictus* are similar. They form a single pair connected by salivary ducts. Each has three lobes: two *lateral* lobes (L) and one *medial* lobe (M). The *lateral* lobe is divided into 2 sections: *proximal lateral* (PL), and *distal lateral* (DL). *Proximal lateral* lobes synthesize enzymes involved in sugar meals, while *medial* and *distal lateral* lobes produce molecules related to blood meals [13;24]. Each lobe produces proteins that can modulate the immune response of the host during blood feeding. Apyrase and α -glucoside, found in the proximal and lateral lobes of the salivary glands of *Aedes*, play a role in modulating the immune response of the host through a platelet aggregation mechanism [25]. The morphology of adult female *Ae. aegypti* and *Ae. The albopictus* salivary gland can be shown in Figure 4.

3.2. In Vitro Analysis of The Human Immune Response against Aedes Salivary Gland Extract

In vitro analysis of human humoral immune response (IgG) in this study was carried out using the ELISA (Enzyme-Linked Immunosorbent Assay) method. The results of human immune response (IgG) from individual sera samples of the neonate, healthy people and DHF patient can be seen in Figures 5 and 6. The negative controls from individual samples have IgG levels zero. This indicates there were no specific antibodies that bind to an antigen. The presence of antibody antigen-binding indicates that sera samples have been exposed by saliva *Aedes* either directly through mosquito exposure in DHF patients, healthy people or through the placenta in the neonate group. Neonate samples detected IgG in low levels, the low level of IgG in the neonate originated from the mother through the placenta during pregnancy. IgG is the only antibody from the mother that can penetrate to the baby through the placenta [26;27].

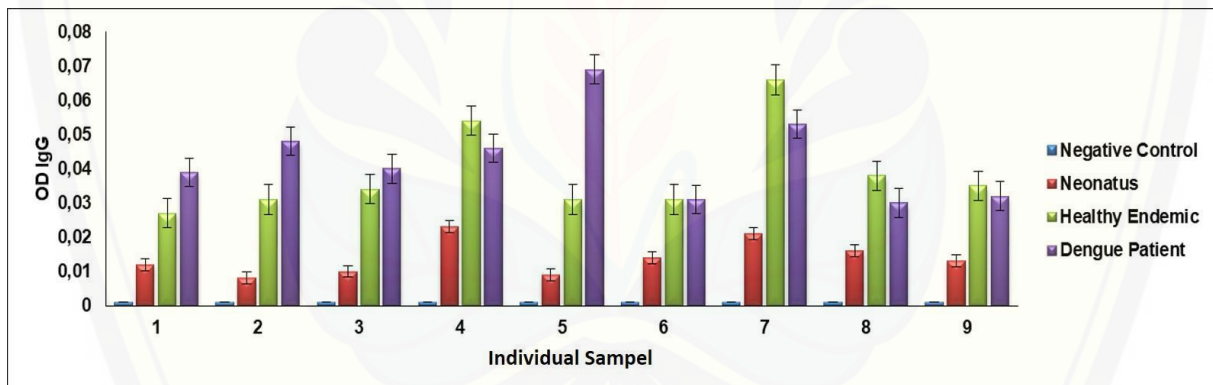


Figure 5. Individual Human Immune Response (IgG) to salivary gland of *Ae. aegypti*.

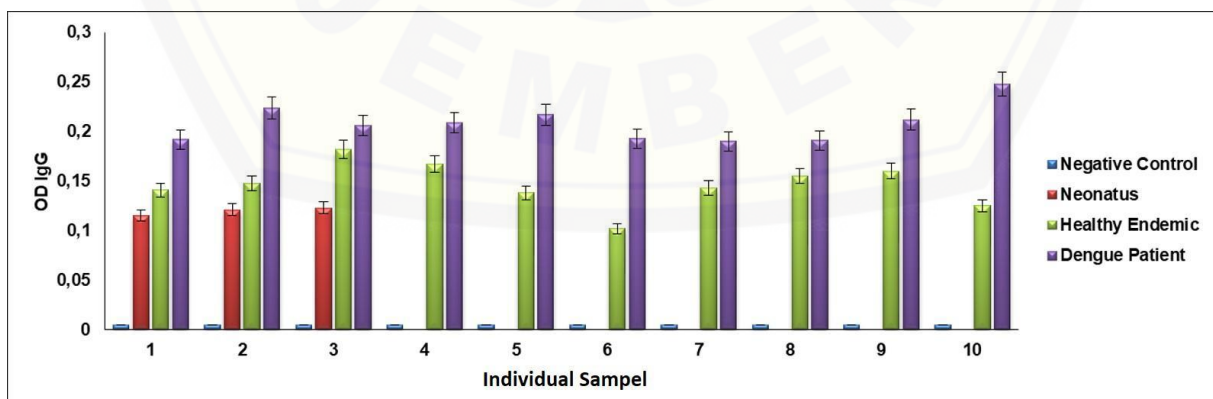


Figure 6. Individual Human Immune Response (IgG) to the salivary gland of *Ae. albopictus*.

The IgG immune response in individual sera samples varied (Figure 5 and 6). The varied immune responses indicate that samples were frequently exposed to mosquitoes. Differences in immune

responses originate from varying exposure intensities [28]. After several instances of blood-feeding, or intensive exposure, the host immune response develops a recognition response that produces antigens in response to the mosquito saliva [29]. A person who is often exposed to saliva *Aedes* will have many specific antibodies, especially IgG [15]. The primary exposure to *Aedes* saliva produces low IgG, while secondary exposure induces a greater response. There was a linear relationship found between IgG production and exposure. This is triggered by increased B cell proliferation, which plays a role in producing IgG [30]. Results indicate that samples from DHF patients and those from healthy people in endemic areas had higher levels of IgG than the neonate samples. These differences likely derive from differing levels of direct exposure to mosquito saliva

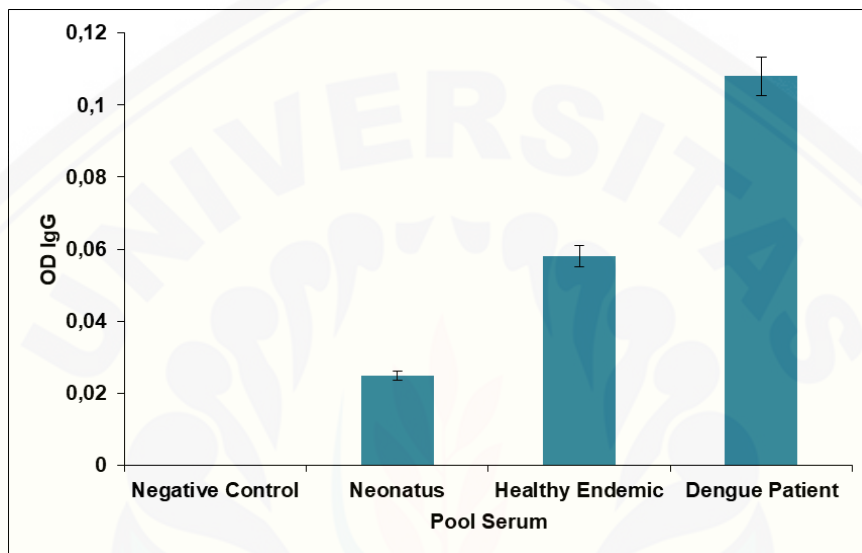


Figure 7. Human Immune Response (IgG) from population sample against salivary gland of *Ae. aegypti*.

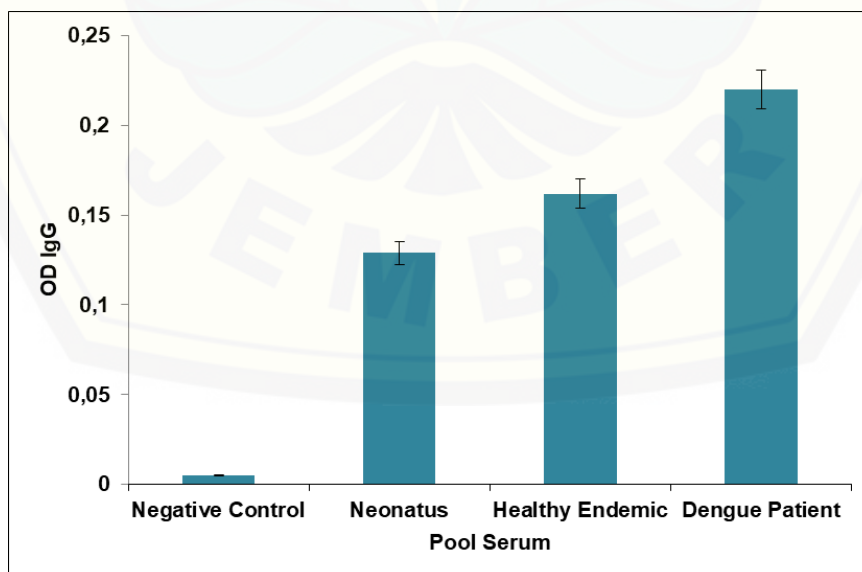


Figure 8. Human Immune Response (IgG) from population sample against salivary gland of *Ae. Albopictus*.

The ELISA analysis revealed a similar immune response across the sample population (Figure 7 and 8). Based on analysis of *independent sample* T test results of the human immune response

against salivary gland extract of *Ae. aegypti* demonstrate the neonate, the healthy and the DHF population had a significance value of 0.00 ($p < 0.05$) as well as the healthy with DHF population. This shows that there was a significant difference of human immune response in each population. While the T-test result of human immune response to *Ae. albopictus* salivary gland extract showed a significance value of 0.094 ($p > 0.05$) in the neonate with the healthy population. This shows that there were no significant differences between these two populations. However, the results of the T test in the neonates and DHF population as well as between the healthy and DHF population have a significance value 0.001 ($p < 0.05$) and 0.032 ($p < 0.05$). This indicates that the human immune response against salivary gland extract of *Ae. albopictus* in neonates and the DHF population as well as between the healthy population and DHF showed significant differences.

The highest immune response was found in DHF patients, followed by that of healthy persons, and finally the neonate samples, respectively. The immune response of dengue patients and healthy people were varied, it was caused that the grade of exposure to each individual was different, therefore each individual has a different response [31]. The healthy residents and DHF patients in endemic areas tend to produce antibodies to salivary exposure to *Aedes* [20]. The density of mosquitoes in an area correlates to IgG host immune response [29]. Results from analysis reveal that endemic people have higher IgG values than the neonate group.

The IgG response is one of the protective mechanisms from the host to respond to the saliva vector as antigen. This mechanism will occur after the host got repeated exposure to mosquito vectors during the blood-feeding process. The healthy people in the endemic area also got exposure more than once, therefore analysis results reveal that endemic people have higher IgG values than neonates. The host immune responses in arthropod-salivates repeated exposure will modulate adaptive immune responses leading to the Th 2. It will activate B cells, thus will form plasma cells to produce specific antibodies (such as IgG) [12;20].

4. Conclusions

The human immune response to an antigen of the salivary gland of dengue vector i.e. *Ae. albopictus* and *Ae. aegypti* shows that dengue patients have the highest value for IgG level over healthy individuals as well as a neonate group. This result indicates that salivary glands extract *Ae. albopictus* and *Ae. aegypti* can modulate the immune system of the host.

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