MICROBIOLOGY INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.11, No.1, March 2017, p 23-27

Available online at
http://jurnal.permi.or.id/index.php/mioline
DOI: 10.5454/mi.11.1.4

Analysis of Human Immune Response against Salivary Glands Protein Extract of *Anopheles sundaicus*. L in Malaria Endemic Area

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Malaria is an infectious disease caused by *Plasmodium*, which is transmitted by *Anopheles* mosquitoes as vectors. Malaria transmission begins when an infected mosquito takes blood meal from healthy human. Mosquitoes will release parasite and components of saliva into the host's body. Saliva contains components (proteins) that affect the host's hemostasis and immune response, such as vasomodulator and immunomodulators. Imunomudulator could act as immunosuppressive factors that can suppress nonspecific immune system of the host and modulate the change of T helper 1 (Th1) toward T helper 2 (Th2) response, which is advantageous for malaria parasite to infect human host. This research wanted to evaluate human immune respons in endemic area against salivary gland protein extract (SGPE) from its major malaria vector *i.e. Anopheles sundaicus (An. sundaicus)*. Analysis of human immune response was conducted quantitatively by ELISA (Enzyme-Linked Immunosorbent Assay) towards IgG from human sera samples after cross reacted with SGPE. The results showed that exposures to *An. sundaicus* were able to induce high levels of IgG. IgG anti salivary proteins of *An. sundaicus* is higher than the levels of IgG anti salivary proteins of *Ae. aegypti*. Furthermore, the age group 11-40 years with the highest bites probability, had the highest IgG levels compared to other age groups.

Key words: *Anopheles sundaicus*, IgG, malaria, salivary

Malaria merupakan penyakit infeksi disebabkan oleh *Plasmodium*, yang ditransmisikan oleh vektor nyamuk *Anopheles*. Transmisi malaria diawali ketika nyamuk yang terinfeksi melakukan *blood feeding* ke manusia sehat. Selama *blood feeding*, nyamuk juga akan melepaskan parasit bersamaan dengan komponen saliva ke tubuh inang manusia, Saliva mengandung kompoten (protein) yang dapat mempengaruhi hemostasis dan respon imun inang seperti vasomodulator and immunomodulators. Imunomudulator dapat bersifat sebagai faktor yang *immunosuppressive* sehingga dapat menekan sistem imun non spesifik serta memodulasi perubahan respon imun spesifik T helper 1 (Th1) ke arah T helper 2. Hal ini sangat menguntungkan parasit sehingga memudahkan infeksinya ke dalam tubuh manusia. Penelitian ini ingin menguji respon imun manusia yang hidup di daerah endemik terhadap ekstrak protein kelenjar saliva (SGPE) dari vektor dominan di daerah tersebut yaitu *Anopheles sundaicus (An. sundaicus)*. Analisis respon imun dilakukan secara kuantitatif dengan ELISA (*Enzyme-Linked Immunosorbent Assay*) dengan mengamati titer IgG dari sampel sera penduduk terhadap SGPE. Hasil penelitian menunjukkan bahwa paparan berulang dari *An. sundaicus* mampu memicu meningkatnya titer IgG. Konsentrai IgG terhadap SGPE *An. sundaicus* lebih tinggi dibandingkan dengan IgG terhadap SGPE *Ae. aegypti*. Kelompok usia 11-40 tahun yang memiliki kemungkinan terpapar gigitan nyamuk lebih tinggi, menunjukkan titer IgG yang tertinggi dibandingkan kelompok-kelompok usia lainnya.

Kata kunci: *Anopheles sundaicus*, IgG, kelenjar saliva, malaria

Malaria is an infectious disease which is caused by *Plasmodium* and spread by *Anopheles* mosquito as the vector. The dissemination of the disease starts when a mosquito that carries *Plasmodium* takes blood meal from healthy human. The mosquito will release saliva components to the host's body. Mosquito's saliva contains components that can influence host's homeostasis including vasomodulator and immunomodulator. Vasomodulatory factors can manipulate the host's vasomodulator mechanism by preventing vasoconstriction, platelet activation and aggregation, and blood coagulation. Immunomodulator factor can supress the host's nonspecific immune system that modulates the changing response from T helper 1 (Th1) to T helper 2 (TH2) (Titus *et al.* 2006). The changing of immune system can be seen through the decrease of IFN-γ production and the increase of Interleukin-4 (IL4). IL4 will influence the proliferation of cell B, which, in turn, will raise humoral antibody. Because

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the role of salivary proteins are mediating easier phatogen transmission, the increasing of humoral antibody against salivary protein will also affect the transmission of phatogen which means exposure against mosquitoe's bite will increase host immune resistancy against its transmitted phatogen (Donovan *et al.* 2007).

Bites from *Plasmodium* infected mosquito can spread malaria. On the other hand, reexposure to sterile mosquito can cause protection, cause the salivary component give the sensitisation effect. In a study conducted by (Morris *et al.* 2001) injection with low dose saliva component can increase the transmission of pathogen, while injection with high dose can give protection. It means that people who live in the endemic area and often get exposure to vector's bite will have protection to pathogen. Recurring exposure causes the changing of immune response like in the normal condition (Th1 response), which are the activation of macrophage and production of Nitric oxide (NO) so that it will be effective to kill parasite (Donovan *et al.* 2007). Because of the increasing production of antibody against salivary antigen (IgG) increase by repeated exposure, it can mediate to block the infection, Therefore people living in endemic area who often get more exposure to vector's bite tend to have more protection from the infection. (Fontaine *et al.* 1995). The measurement of anti-salivary protein antibody (IgG) can be used as biomarker of the exposure to *Anopheles* mosquito. In Indonesia, especially in endemic area of Bangsring village, Banyuwangi, there has not been any analysis of immune response towards *Anopheles* saliva protein in the population. Analysis of host's immune response towards salivary gland protein of *An. sundaicus* is an important step to determine if human exposure to mosquito bite is related to the production of specific antibody. If such a relationship can be proven, this antibody might be used as a biomarker of the exposure.

MATERIALS AND METHODS

Landing Collection, Identification and Rearing *An. sundaicus.* *Anopheles* mosquito was reared in mosquito cage at the zoology laboratory, Biology Department, Faculty of Mathematic and Natural Science, Universitas Jember. Rearing process started with collecting (landing collection) mosquitoes from their habitat in Bangsring village at Wongsorejo, Banyuwangi. *Anopheles* larva was collected from lagoons near the coast, while adult *Anopheles* mosquitoes were gathered from around livestock pens near people's house. The Anopheles mosquitoes identified in the laboratorium based on the book of determination key of Insect. The rearing started from adult mosquitoes that were kept at room temperature (25-28 ^{o}C) and given 10% sucrose solution diet, and periodically were given the body of wistar rat as the source of blood. The mosquitoes were given this diet since the first day. Humidity was kept by wrapping the mosquito cage with wet fabric.

Extracting *An. sundaicus* Salivary Gland Protein. Salivary gland was isolated by micro dissection, with the addition of lysis buffer (1:1 ratio). Then, the sample was homogenized, sonicated for 30 min by using water sonicator, centrifuged with 12690 rpm for 15 min at 4 ^{o}C. Then, supernatant was taken and kept at 80 ^{o}C. Salivary protein was concentrated using epi membrane centrifuged at 10000 rpm at 4 ^{o}C for 30 s. The concentrated protein was kept at 80 ^{o}C until further use.

Preparing Blood Serum. Serum samples of the were taken from healthy people's blood at endemic area in Bangsring, Banyuwangi. The volunteers were grouped based on their age, children (<10 year old), adult (11-40 year old), and old (>40 year old). Blood sample was taken from the branchial vein in the upper arm. Three mL blood was taken and placed in vacutainer without heparin. Then, it was kept for 15 to 45 min. After that, upper transparent layer was taken and centrifuged at 27 ^{o}C, 3200 rpm for 10 min. The serum from the supernatant was then kept at the temperature of -80 ^{o}C.

Indirect ELISA (Enzyme Linked Immuno-Sorbent Assay). The plate was coated with 5 µg mL^{-1} (50 µL well^{-1}) *An. sundaicus* SGPE, which has been diluted with 0.1 M natrium bicarbonate buffer (pH 9.6). Coating was performed overnight at 4 ^{o}C. Then, the plate was washed with 250µL PBS-T (pH 7,4). The plate was blocked with 200µL blocking buffer (PBS-T ; 1% *Bovine Serum Albumine*) for 2 h at 37 ^{o}C. Serum was diluted with 1:25 ratio (50 µLwell^{-1}) and incubated at 37 ^{o}C for 1 h. Then, 50µl *Horse Radish Peroxidase (HRP)-conjugated Rabbit anti human* IgG (1:5.000) was added and incubated for 1 h at 37 ^{o}C. After that, 50 µL *tetra methyl benzidine* substrate was added and incubated for 10 min at room temperature. Then 50µL 1M H_2SO4 was added to *stop the reaction.* The level of IgG was determined using ELISA reader set at 450nm. The Control well also applied with the same methods, but without adding serum into the well, it substitutes by blocking buffer.

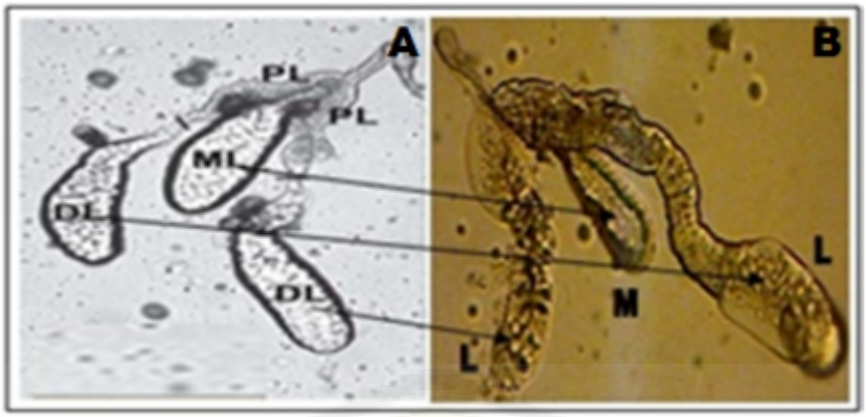

Fig 1 Salivary gland of female *Anopheles*. Note: PL: Proximal Lobe, DL: Distal Lobe, ML: Medial Lobe. A: *Anopheles sp.* salivary gland. (Jariyapan *et al.* 2007). B: the result of *An. sundaicus* salivary gland isolation (LW scientific microscope, 400 x magnifications, Optilab camera).

Data Analysis. The data obtained in this research were analyzed using softwares Graphpad Prism 5.0 and SPSS, with one way anova ($p<0.05$) and Duncan's ($p<0.05$) tests.

RESULTS

Salivary Gland and Salivary Gland Protein Extract from *An. sundaicus.* There are 800 pairs of female *An. sundaicus*' salivary gland were isolated by micro dissection technique (Bruce 1980). Female *Anopheles* only had one pair of salivary gland which was located in each side of esophagus at the anterior thorax (Wright 1969). One salivary gland consisted of three lobes, two lateral lobes and one medial lobe. Salivary duct connects medial lobe and salivary pump, which is located near hypopharynx. Lateral lobes are divided into proximal, intermediate, and distal area (Dhar and Kumar 2003). *An. sundaicus* salivary gland can be seen in Figure 1. The total amount of SGPE extracted from *An. sundaicus* was 4.2 mg mL^{-1}.

Human IgG level towards SGPE from *An. sundaicus.* This research used two different mosquito salivary glands protein extracts (SGPEs), which were *An. sundaicus* SGPE and *Aedes (Ae.) aegypti*. The result of IgG measurement can be seen in Figure 2. The comparison between two antigens was done to determine the level of IgG introductory to SGPE. Figure 2 shows higher OD score was observed on detection of IgG against *An. sundaicus* SGPE than *Ae. aegypti* SGPE.

DISCUSSION

In this research, there were 2000 *Anopheles* mosquitoes were collected from the field in 2014. The species of *Anopheles* mosquitoes which found based on the result of landing collection consisst of: *An. annularis, An. vagus, An. subpictus, An. idenfinitus, An. barbirostris,* and *An. sundaicus*. The most dominant species collected was *An. sundaicus*. This result was consistent with the previously published results (Lyimo and Takken 2008), which stated thatat least seven species of *Anopheles* had been found in the Bangsring village, in which the most dominant was *An. sundaicus*. SGPE consists of many different proteins, some of which can be conserved into genus and even family level (Fontaine *et al.* 2011).

The high respond of IgG anti SGPE from *An. sundaicus* in Figure 2 is due to the place where the serum was taken was endemic area of malaria with high population of *An. sundaicus*. It is in line with previous research (Shinta *et al.* 2003) which stated that Bangsring village in Wongsorejo; Banyuwangi was endemic of malaria, in which *An. sundaicus* was the primary vector. The level of *Ae. Aegypti* SGPE - reactive IgG was also high. Most probably, this was because *Ae. aegypti* is ubiquitousin Indonesia, as shown by the high case of dengue fever all over the country(Depkes RI 2004).

The high level of *Ae. aegypti* SGPE - recognising IgG might also be because whole protein extract was used. Tthere is high chance that plenty of homology occur in the salivary proteins ofmosquitos in the Cullicidae family, to which group both *Ae. aegypti* and *An. sundaicus* belong to (Fontaine *et al.* 2011). In this research, the grouping was based on age. There was no level of IgG or the IgG level was zero in the control group. There was no antigen in the control group so that antibody within the serum could not attach to its specific antigen. However, in the neonates group

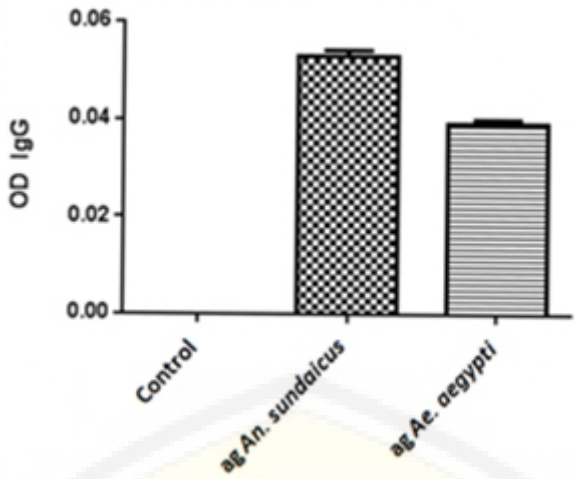

Fig 2 Graph of IgG level based on salivary protein antigen (Ag). There is significant differences between each group (ANOVA P=0.000 < 0.05), and the higher salivary anti protein IgG level is *An. sundaicus* antigen (Duncan p=0.000 < 0.05).

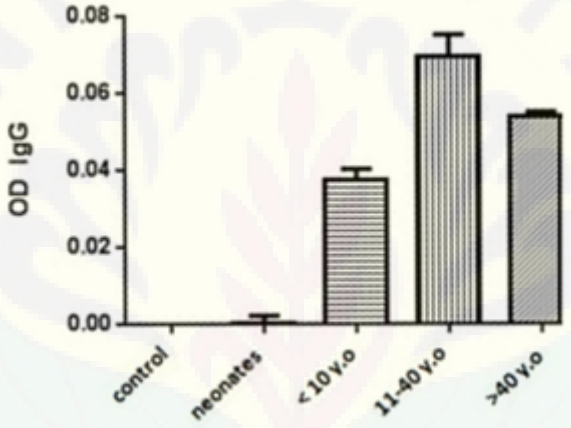

Fig 3 The result of IgG measurement based on age group. The five experimental groups show significant difference (ANOVA p=0.000 < 0.05). Then, the highest level of anti salivary protein was in the group of 11-40 years old (Duncan p=0.00 < 0.05).

showed low OD score. It was assumed that there was anti salivary protein in the serum from the mother. The group of 11-40 year old showed the highest level of IgG compared to the other groups. The high OD score in group of 11-40 year old might due to two reasons which were the influence of age which could influence the activation of T memory cell and influence the production of specific antibody (Lyimo *et al.* 2008). At the active age group (11 to 40) the immune response was more mature so that the IgG level was high while the group of >40 year old the level of antibody production had decreased. On the other hand, in the group of <10 year old the immune system is still developing (Bratawidjaja and Rengganis 2014). The second possibility was supported by the result of the questionnaire which had been gathered before the blood sampling. Most of people in Bangsring village who were 11 to 40 years old had activities outside their home at night. This was also related to the behavior of *An. sundaicus* which was more active at night (nocturnal) (CDC 2010) and the species of *An. sundaicus* was found more outside than inside of house (Mardiana *et al.* 2003). The measurement of IgG based on age group can be seen in Figure 3.

A person who is often exposed to mosquito bite will have higher anti salivary protein IgG level than a person who is rarely exposed to mosquito bite. The result of this research was consistent with the previous research, stating that people living in malaria endemic area who were often exposed to anopheles saliva would express higher immunity level by producing antibody in the form of anti salivary protein IgG (Waitayakul *et al.* 2006). The data from questionnaire states that volunteers younger than 10 years old spent most of their night at home. They used anti-vectorial such as insecticide netting, body lotion, and mosquito coil,

which could prevent them from the mosquito bites.

From our data, therefore, it is indicated that there is correlation between antibody response against SGPE with exposure to *An. sundaicus*. High level of anti-SGPE antibody against *An. sundaicus* in sera samples from human living in endemic area can be used as a potential source of indicators of exposures to *An. sundaicus*. Even in comparison to anti-SGPE antibody against *Aedes aegypti,* which is also in Indonesia, our results demonstrated that the level of antibody against *An. sundaicus* was still higher. Serum analysis also supported the hypothesis that anti SGPE-antibody level increased with the probability of exposure to mosquito bites.

ACKNOWLEDGEMENT

We are very grateful to Ministry of Research, Technology, and Higher Education especially" Hibah Pascasarjana" for providing financial support for this study.

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