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 response 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 komponen (proteин) yang dapat mempengaruhi hemostasis dan respon imun inang seperti vasomodulator dan immunomodulators. Imunomodulator 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 infeksiya ke dalam tubuh manusia. Penelitian ini mengingat 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. Konsentrasi 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 immuno-
the role of salivary proteins are mediating easier pathogen transmission, the increasing of humoral antibody against salivary protein will also affect the transmission of pathogen which means exposure against mosquito's bite will increase host immune resistancy against its transmitted pathogen (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 especially in endemic area of Bangsring village, Banyuwangi. There has not been any analysis of exposure to _An. sundaicus_.

**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 °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 °C. Then, supernatant was taken and kept at 80 °C. Salivary protein was concentrated using epi membrane centrifuged at 10000 rpm at 4 °C for 30 s. The concentrated protein was kept at 80 °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 °C, 3200 rpm for 10 min. The serum from the supernatant was then kept at the temperature of -80 °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 °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 °C. Serum was diluted with 1:25 ratio (50 µLwell⁻¹) and incubated at 37 °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 °C. After that, 50 µL tetra methyl benzidine substrate was added and incubated for 10 min at room temperature. Then 50µL 1M H₂SO₄ 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.
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 consists of: An. annularis, An. vagus, An. subpictus, An. identifinitus, 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 that at 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 ubiquitous in 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. There is high chance that plenty of homology occur in the salivary proteins ofmosquitos in the Culicidae 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
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.

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**REFERENCES**


