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Detection of immunogenic proteins from *Anopheles sundaicus* salivary glands

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ABSTRACT

Introduction: The saliva of mosquitoes has an important role in the transmission of several diseases, including malaria, and contains substances with vasomodulating and immunomodulating effects to counteract the host physiological mechanisms and enhance pathogen transmission. As immunomodulatory components, salivary gland proteins can induce the generation of specific IgG antibodies in the host, which can be used as specific biomarkers of exposure to *Anopheles sundaicus*. The objective of this study was to identify immunogenic proteins from the salivary glands of *Anopheles sundaicus* by reaction with sera from individuals living in malaria-endemic areas who are thus exposed to *Anopheles* mosquitoes. **Methods:** IgG antibodies targeting salivary gland proteins in serum samples from individuals living in malaria-endemic areas were measured by enzyme-linked immunosorbent assay (ELISA). Sera from healthy individuals living in non-endemic areas were used as negative controls. Determination of the presence of salivary gland immunogenic proteins was carried out by western blotting. **Results:** Sixteen bands appeared in sodium dodecyl sulfate polyacrylamide gel electrophoresis, with molecule weights ranging from 22 to 144kDa. Among the exposed individuals, IgG responses to salivary gland proteins were variable. Protein bands with molecular weights of 46, 41, 33, and 31kDa were the most immunogenic. These immunogenic proteins were consistently recognized by pooled serum and individual samples from people living in malaria-endemic areas but not by negative controls. **Conclusions:** These results support the potential use of immunogenic proteins from the salivary glands of *Anopheles* as candidate markers of bite exposure or in malaria vaccines.

Keywords: *Anopheles sundaicus*. Salivary glands. Immunogenic proteins. Antibody.

INTRODUCTION

Malaria is a mosquito-borne disease that has major health implications worldwide, with an estimated 207 million individuals affected by malaria and 627,000 malaria-related deaths reported. In 2013, 104 countries and territories, including Indonesia, had malaria, which was considered endemic; an estimated 3.4 billion people are at risk of contracting malaria[1].

Malaria is caused by five species of parasites from the genus *Plasmodium* and naturally spread from one person to another by female *Anopheles* mosquitoes. There are about 400 different species of *Anopheles* mosquitoes, but only 30 of these are vectors of major importance[1]. *Anopheles sundaicus* is one of the most important malaria vectors in Indonesia, particularly in coastal area of Java and the Sumatra islands. The distribution of *Anopheles sundaicus* also includes countries in Southeast Asia and India[2][3].

The important role of *Anopheles* mosquitoes as malaria vectors is supported by the presence of salivary glands in female *Anopheles* mosquitoes. *Anopheles* mosquito salivary glands secrete substances that can enhance the transmission of *Plasmodium*[4]. These substances inhibit hemostatic processes and modulate the host immune response (immunosuppressive) at site of biting, allowing the mosquito to feed on blood successfully[5][6]. These changes at the site of the bite, owing to the effects of salivary substances, would benefit the pathogen, permitting infection without any resistance and thus enhancing infectivity in the vertebrate host[7].

Injection of saliva into the host's skin also induces the production of antibodies against salivary proteins[8]. The immunogenicity of salivary proteins has been demonstrated in previous studies, which showed that salivary proteins can induce the host immune response by producing immunoglobulin G (IgG) and immunoglobulin E (IgE) via hypersensitivity reactions[9]. The presence of a specific IgG antibody response against whole saliva extracts (WSEs) of *Anopheles*

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mosquitoes can be measured by enzyme-linked immunosorbent assays (ELISAs) in children and adults exposed to mosquitoes bites[10][11]. Additional studies have shown that the IgG response can recognize specific salivary proteins[12][13]. Therefore, salivary proteins (e.g., gSG6) that are able to generate specific IgG antibodies have been developed as serological indicators or biomarkers of exposure to malaria vectors[14].

Immunoglobulin G antibodies against *Anopheles* salivary proteins in the host may be associated with protection against malaria. In malaria-endemic regions, populations exposed to uninfected *Anopheles* bites repeatedly over the years may develop an anti-saliva immune response by producing specific antibodies in the presence of interleukin (IL)-10. These specific antibodies will neutralize some salivary proteins of vectors, thus causing micro-environmental changes at the site of the mosquito bite and ultimately affecting malaria transmission. Therefore, in asymptomatic patients with malaria, the level of IgG antibodies targeting anti-salivary gland sonicates (SGSs) from *Anopheles darlingi* are higher than those in symptomatic patients and healthy individuals[15]. Exposure to *Anopheles* mosquito bites and *Plasmodium* infections, which persist for a long time, affect the development of the immune response to parasites and salivary vectors, thereby influencing the number of parasites and the host response[16]. Thus, salivary components could be effective vaccine candidates for reducing the morbidity of vector-borne diseases through combination with other malaria vaccine candidates to protect against severe malaria[7]. Further characterization of salivary proteins and the immune response is needed to identify salivary proteins involved in protection against malaria; the first step in this process is determination of the immunogenicity of the salivary proteins. Many studies have been conducted to identify and characterize immunogenic salivary proteins from malaria vectors in Africa; however, malaria vectors in Asia, particularly *An. sundaicus*, have not been studied[8][17][18].

Therefore, in this study, we measured the levels of IgG antibodies targeting salivary proteins in serum samples from individuals living in malaria-endemic areas using salivary glands extracts (SGEs) from *An. sundaicus*. Based on the IgG antibody response to salivary proteins, we identified the immunogenic proteins contained within *An. sundaicus* SGEs.

METHODS

Anopheles mosquitoes and isolation of salivary glands

The adult female *Anopheles sundaicus* mosquitoes used in this study were collected from Bangsring village, Wongsorejo District, Banyuwangi Regency in East Java province using aspirators. In this area, *Anopheles sundaicus* is the dominant vector because its population is larger than that of other common species, such as *Anopheles vagus*, *Anopheles subpictus*, *Anopheles barbirostris*, and *Anopheles annularis*[19]. These mosquitoes were maintained in the insectariums of the Zoology Laboratory of Biology Department, Faculty of Mathematic and Natural Sciences, Jember University at 28°C with 60-70% relative humidity and 10% sucrose. The salivary glands were dissected using fine entomological needles under a stereoscopic microscope at 4× magnification and collected into a microcentrifuge tube containing a small amount of phosphate-buffered saline [(PBS); pH 7.2] and phenylmethylsulfonyl fluoride (PMSF) as protein inhibitors. The salivary glands were stored at -80°C until use.

Salivary gland extraction and protein quantification

One hundred salivary glands pairs in PBS and PMSF were thawed on ice and mixed in 1:1 lysis buffer containing 1.5mM $MgCl_2$, 10mM Tris HCl, 10mM NaCl, 1% Nonidet P-40, and 2mM ethylenediaminetetraacetic acid (EDTA) NaOH[20]. The mixture was homogenized using a micropestle and sonicated using a water sonicator for 30 min. After centrifugation (12,600rpm for 15 min at 4°C), the resulting supernatant was collected and concentrated using a spin concentrator (cut-off of 10kDa; Corning) and centrifugation (10,000rpm for 30s at 4°C). The protein concentrations of SGEs were determined using a nanophotometer (Nanophotometer Implen P 360, Germany). Salivary gland extracts were then diluted in 0.1M bicarbonate buffer (pH 9.6) to obtain a protein concentration of 1µg/µL for enzyme-linked immunosorbent assay (ELISA)[11].

Human serum samples

Twenty serum samples were collected from healthy adult residents living in the location at which we collected *An. sundaicus*, i.e., Bangsring village, Wongsorejo District, Banyuwangi Regency in East Java province, to detect antibodies against *An. sundaicus* salivary gland proteins. Seven serum samples from healthy adult residents living in non-malaria regions were used as negative control. The human subjects protocol for this study was approved by the Ethical Committee of Medical Research, Medical Faculty, Brawijaya University.

ELISA

To optimize the working conditions for ELISAs, checkerboard titration was carried out using *An. sundaicus* SGEs at 1, 2, and 4µg/mL and serially diluted serum samples (1:25, 1:50, and 1:100) from healthy individuals living in the malaria-endemic region. ELISA was performed as described by Fontaine et al.[11]. Based on the results of checkerboard titration, microtiter immunoplates (SPL Life Sciences, Korea) were coated with 4µg/ml (50µL/well) of *An. sundaicus* SGEs diluted in 0.1M bicarbonate buffer (pH 9.6) overnight at 4°C. Three washes were carried out using PBS-T (PBS, pH 7.4, containing 0.05% Tween-20; Nacalai, Japan) between each incubation. The plates were blocked for 2h at 37°C with 200µL of blocking solution buffer consisting of PBS, 0.05% Tween-20, and 1% bovine serum albumin (BSA; SERVA, Germany). Serum diluted 1:25 in blocking buffer was added (50µL/well) into duplicate wells and incubated at 37°C for 1h. After washing, 50µL of horse radish peroxidase (HRP)-conjugated rabbit anti-human IgG (1:5,000; Surmodics, USA) diluted in blocking buffer was added, and the plates were incubated at 37°C for 1h. Enzyme activity was detected by incubation with 50µL of tetramethylbenzidine substrate (KPL, USA) for 30 min at room temperature. Fifty microliters of 1M H_2SO_4 was added to stop the reaction.

The optical density (OD) at 450nm was determined with a microplate reader (Bio-Rad, USA). A pool of five serum samples from individuals living in Bangsring village, which all exhibited high levels of antibody responses against *An. sundaicus* SGEs based on the ELISA optimization test, was used as a positive control. The negative control was individual serum samples from individuals who had never been exposed to *An. sundaicus* bites. The level of IgG antibodies was expressed as the adjusted OD (aOD), which was calculated for each serum sample duplicate as the mean OD value for wells with SGEs minus the OD value of the control wells, i.e., without SGEs.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the methods described by Jariyapan et al.[21]. Briefly, ten salivary gland pairs were mixed 1:2 in 1× SDS gel loading buffer and boiled in a water bath for 5 min. The mixtures were then loaded on 12% SDS polyacrylamide gels. To visualize the bands, gels were stained with Coomassie Brilliant Blue (CBB). A molecular weight marker (Nacalai) was loaded on each gel to identify the proteins in SGEs.

Western blotting of salivary gland proteins

Gels from SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes (MACHEREY-NAGEL, Germany) using semidry blotting (Bio-Rad) for 1h at 100mA. The membranes were blocked by incubation in 5% nonfat dry milk dissolved in (blocking buffer) for 1h at room temperature. After washing with -Tween 0.05% (T) three times, membranes were incubated overnight at 4°C with serum samples diluted 1:20 in blocking buffer. Subsequently, membranes were incubated with alkaline-phosphatase goat anti-human IgG secondary antibodies at a dilution of 1:2,000 for 2h at room temperature after three washes in TBST. Nitro blue tetrazolium-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) Phosphatase substrate was used for color development. To estimate the protein size, prestained broad-range molecular weight markers (9-200 kDa; Nacalai) were used.

RESULTS

Protein profiles of *Anopheles sundaicus* salivary glands

Salivary glands of female mosquitoes consist of three lobes: the two lateral lobes and the medial lobe, which is attached to the salivary duct **(Figure 1)**. The lateral lobes are longer than medial lobe and are formed by the proximal, intermediate, and distal regions, whereas the median lobe is formed by a short neck region and distal region. Based on the results of SDS-PAGE, we found that the protein profiles of salivary glands from female *An. sundaicus* consisted of 16 bands with molecular weights ranging from 24 to 138kDa **(Figure 2)**. Among these 16 bands, there were some major bands observed at estimated molecular weights of 144, 120, 91, 62, 46, 40, 36, 33, 31, 26, and 22kDa.

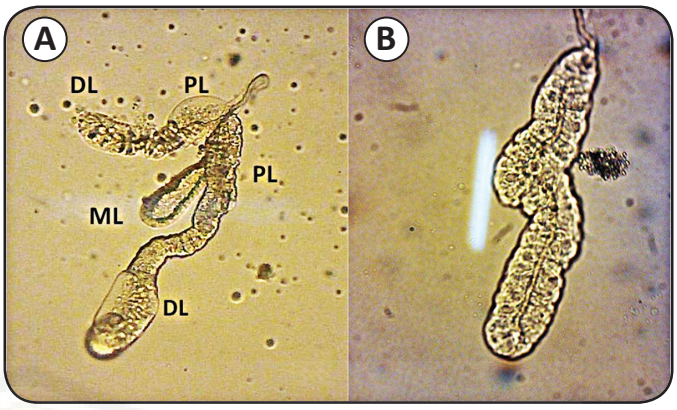

FIGURE 1 - Single salivary glands from adult *Anopheles sundaicus* mosquitoes. A) A female salivary gland. B) A male salivary gland. DL: distal region of lateral lobe; **PL:** proximal region of the lateral lobe; **ML:** median lobe. (Nikon stereoscopic microscope at 4× magnification).

Levels of anti-salivary gland protein IgG antibodies

ELISA was used to detect and measure the levels of anti-salivary gland protein IgG antibodies. The levels of antibodies against salivary proteins in sera from healthy individuals living in Bangsring village were variable. These variations could be influenced by the intensity of exposure to mosquito bites and mosquito density[11]. There were three serum samples with low OD values (less than that of the negative control) among the 20 serum samples. Therefore, these three samples were not used for detection of immunogenic proteins by western blotting.

Immunogenic proteins found in *Anopheles sundaicus* salivary glands

The results of western blotting showed the presence of several immunogenic proteins with molecular masses of 56, 46, 41, 38, 36, 33, 31, and 26kDa **(Figure 3)**. These results were obtained by using sera from individuals living in Bangsring village, and three repetitions were performed. Among the protein bands recognized by the anti-salivary protein antibody, the most immunogenic proteins had molecular masses of 46, 41, 33, and 31kDa (these protein bands were present in 10-12 serum samples). These immunogenics proteins were consistently recognized by individual responses from 17 serum samples and by pooled serum from individuals living in malaria-endemic area (Bangsring village) as a positive control **(Figure 4A)**. The human antibody response to salivary proteins from female *Anopheles* mosquitoes is also specific to *Anopheles* mosquito bites[12] [17]. Our result showed that immunogenic proteins were not detected by western blotting in pooled serum samples from seven individuals living in non-malaria-endemic areas as a negative control **(Figure 4B)**.

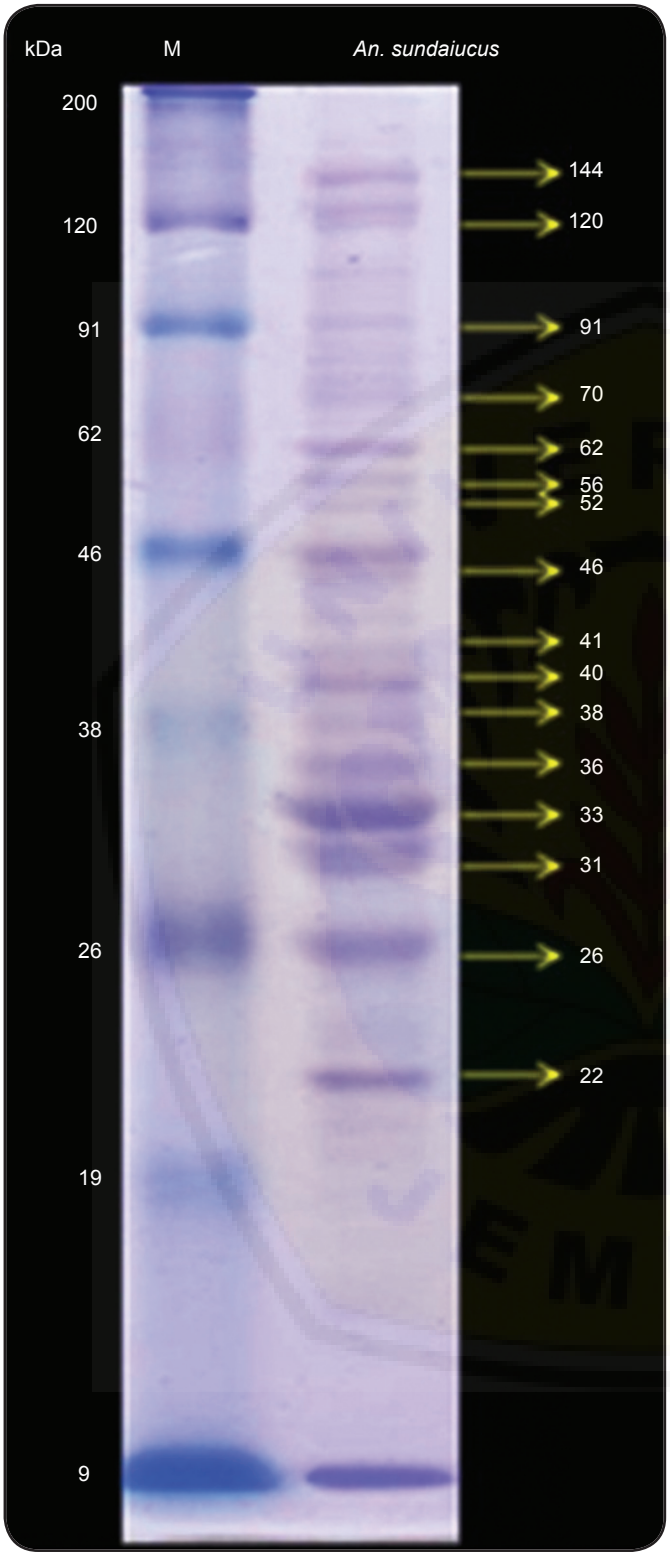

FIGURE 2 - Salivary gland proteins from female *Anopheles sundaicus* mosquitoes, separated using 12% SDS-PAGE (right lane) and stained with Coomassie Blue. Molecular weight markers are shown in the left lane. kDa: Kilodalton; **M:** Marker**; An.:** *Anopheles*; **SDS-PAGE:** sodium dodecyl sulfate polyacrylamide gel electrophoresis.

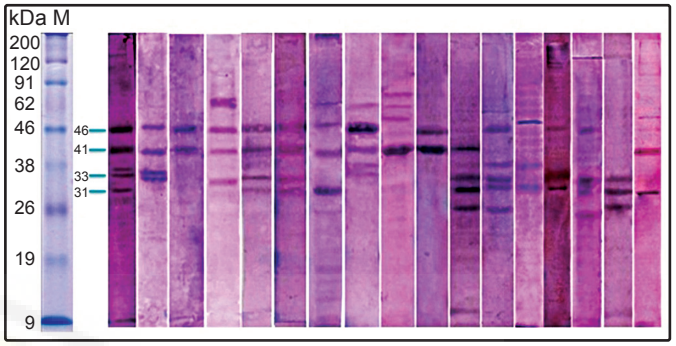

FIGURE 3 - Western blotting using *Anopheles sundaicus* salivary gland proteins detected with human sera from individuals living in a malaria-endemic area. The most highly immunogenic proteins are shown by intense bands at 46, 41, 33, and 31kDa. kDa: Kilodalton; **M:** Marker.

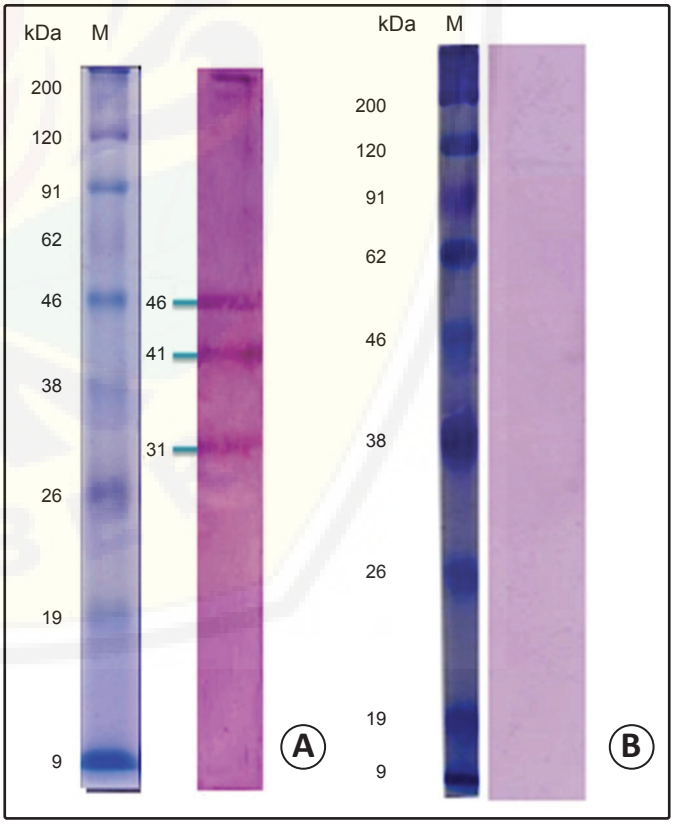

FIGURE 4 - A) Western blotting of anti-salivary gland protein IgG antibodies in a pool of human serum samples from individuals living in a malaria-endemic area (positive control)) and B) individuals living in a non-malaria endemic area (negative control). kDa: Kilodalton; **M:** Marker**; IgG:** immnoglobulin G.

DISCUSSION

The protein profiles of *Anopheles sundaicus* salivary glands have not been studied before; however, some studies have reported the protein profiles of other *Anopheles* species, i.e., *Anopheles dirus*, *Anopheles gambiae*, and *Anopheles stephensi*[12] [17] [18] [21]. A previous study by Cornelie et al.[17] demonstrated the expression of 20 proteins from the salivary glands of *An. gambiae*, which were clearly detected in silver stained gels. Some of these proteins had the same molecular weights as proteins from salivary glands of *Anopheles sundaicus*, i.e., 61-63, 52-54, 41-44, 38-39, 31-34, and 26kDa, similar to the major protein bands detected from the salivary glands of *An. dirus*[21]. This result may be explained by the presence of several proteins in the salivary glands of *Anopheles* that are conserved at the genus level[7]. Some proteins families are found in all *Anopheles* species; these are called genus-specific anopheline secreted proteins and include apyrase/5′ nucleotidase, antigen 5/gvag, GE-rich/30kDa, long and short form D7, mucin/13.5kDa, SG3, SG7, SG10, and hypothetical 6.2-kDa protein families[18]. Potential proteins having molecular weights of 61-63kDa include apyrase/5′ nucleotidase, which is involved in the blood feeding process through degradation (hydrolysis) of adenosine diphosphate (ADP) and adenosine triphosphate (ATP) to adenosine monophosphate (AMP), a mediator of platelet aggregation, inflammation, and neutrophil activation[5] [22] [23]. Long form D7 protein families have molecular weights ranging from 33 to 34kDa, and GE-rich/anti-platelet family proteins have molecular weights around 30kDa; these proteins are also involved in the blood feeding process[22] [23].

The salivary gland proteins of *An. sundaicus* could be recognized by IgG antibodies in human sera from individuals living in Bangsring village, particularly proteins with molecular masses of 56, 46, 41, 38, 36, 33, 31, and 26kDa. Among these proteins, those with molecular masses of 46, 41, 33, and 31kDa were the most immunogenic. This result showed that exposure to mosquito bites in individuals living in malaria-endemic areas, could induce the immune response by producing salivary protein-specific IgG antibodies[10] [12]. Indeed, the IgG antibody response against salivary gland proteins can be influenced by exposure to mosquito bites. In individuals with different levels of exposure to *An. gambiae* bites, the levels of anti-salivary gland protein IgG antibodies are positively related to the intensity of exposure to *Anopheles* mosquito bites. The IgG response increases significantly with the increase in *Anopheles* exposure, as evaluated using conventional entomological methods during the transmission season[10]. The results of a study conducted by Fontaine et al. also demonstrated a positive correlation between the average IgG response to SGEs of *Aedes caspius* and *Aedes caspius* density, which was affected by changes in the season and the ecological environment[18]. The level of the IgG response increased significantly during peak exposure to *Ae. caspius* in September and declined to baseline values within 4 months (January)[11]. These results also showed that IgG responses induced by mosquito saliva antigens persisted for only a short time. Consistent with this, in a study in Senegal, children who experienced low and moderate exposure exhibited decreased antibody concentrations clearly after more than 3 months[10]. Another study showed that the IgG antibody response to salivary gland proteins appearing after exposure to mosquito bites lasts 3-6 weeks[8]. Although the duration of the IgG response to salivary gland proteins may be brief (between 1 and 4 months), this response can detect specific proteins of the salivary gland, i.e., gSG6, in children exposed to very infrequent *Anopheles* bites. According to this study, immunogenic proteins from the salivary glands of *Anopheles* could be developed as immunoepidemiological markers to assess the risk to very infrequent *Anopheles* bites in the context of changes in seasons, different environmental conditions, and travel[13].

The observed immunogenic proteins with molecular weights of 41 and 46kDa could be members of the SG1 family or TRIO proteins, which have molecular weights ranging from 40 to 48kDa[23] [24]. TRIO is a multidomain protein that binds the lymphocyte activating receptor transmembrane tyrosine phosphatase (PTPase) and contains a protein kinase domain; however, the function of this protein still needs to be determined[22]. Our results also showed that proteins with molecular weights of 41 and 46kDa were the most immunogenic because these proteins were recognized by anti-salivary gland protein antibodies from 12 serum samples. According to a previous study, TRIO protein is antigenic in four species *Anopheles*, i.e., *Anopheles gambiae*, *Anopheles arabiensis*, *Anopheles stephensi*, and *Anopheles albimanus*; the results of mass spectrometric analyses showed that this protein is conserved within the Cellia subgenus[18]. TRIO protein is one of several antigenic proteins, in addition to gSG6, gSG1b, SG5, and long form D7, that are over expressed in salivary glands infected with *Plasmodium falciparum*. Therefore, TRIO proteins may be involved in malaria transmission and may represent a new candidate biomarker for infected *Anopheles* bites[25].

The observed immunogenic protein with a molecular weight of 33 kDa could be a long form D7 family protein; these proteins range in molecular weight from 33 to 36 kDa[23]. D7 proteins are also members of the odorant-binding protein superfamily (ODP) and are found in the salivary glands of blood-sucking insects, such as mosquitoes, sand flies, and Culicoides[26]. This protein has two types, i.e., the short form, which is only found in mosquitoes, and the long form, which can be found in mosquitoes and sand flies[23] [24]. D7 proteins have functions related to the binding of one or more agonists hemostasis and the blood feeding process[26]. Some D7 proteins, i.e., D7r1, D7r2, D7r3, D7r4, and long form D7, have been shown to bind with biogenic amines compounds, such as serotonin, histamine, and norepinephrine, thus becoming antagonistic to vasoconstrictors and affecting platelet aggregation and induction of pain[27].

According to a previous study[26], which analyzed the salivary glands of *An. darlingi* by mass spectrometry, the 31-kDa protein identified in our study could be a member of the 30-kDa allergen protein family or the GE-rich/anti-platelet family. Members of the 30-kDa allergen protein family from the salivary glands of *Ae. aegypti* were shown to be associated with an allergic reaction to mosquito bites involving IgE and lymphocyte-mediated

hypersensitivity[28]. This protein, also called aegyptin, has been shown to inhibit platelet aggregation induced by collagen in humans and to impede granule secretion. Aegyptin recognizes the specific binding sites of glycoprotein IV, integrin α2β1, and von Willebrand factor and can the refore prevent the interaction between collagen and these major ligands[29]. Anophelin anti-platelet protein (AAPP) is a protein found in the salivary glands of *An. stephensi* that is homologous to the 30-kDa *Aedes aegypti* allergen[23]. This protein is also a member of the GE-rich family of proteins. AAPP has been shown to block the adhesion of platelets to collagen via direct binding to collagen, subsequently inhibiting the increase in intracellular calcium ion concentration[30]. Therefore, this protein has an important role in blood feeding by inhibition of the hemostatic process.

This study is the first to show the protein profile and immunogenic proteins from the salivary glands of *An. sundaicus*, one of the major malaria vectors in South Asian countries. Several studies have described anti-salivary protein antibody responses that recognize some proteins of *Anopheles* salivary glands, particularly malaria vectors in Africa[17] [18]. However, few studies have investigated immunogenic proteins from salivary glands of *Anopheles* mosquitoes in Asia[12]. Therefore, this analysis herein provides important insights into immunogenic proteins from *Anopheles* mosquitoes in Asia.

Several immunogenic proteins from different *Anopheles* species have been shown to have similar molecular weights, suggesting that these proteins could be conserved at the genus level[18]. The same immunogenic proteins in different *Anopheles* mosquitoes, such as SG6, may trigger wide cross-reactivity between *Anopheles* species[31]. This universal characteristic of immunogenic proteins may improve their applicability as malaria vaccines in different regions. This fact also supports the use of this protein as an indicator of exposure to several *Anopheles* mosquitoes in different areas. The results presented in this study represent the initial step for identification and further characterization of immunogenic salivary proteins from *An. sundaicus*, which is necessary to determine their role in malaria transmission and blood feeding processes.

In conclusion, the present study showed that there were four major immunogenic proteins, i.e., 46, 41, 33, and 31kDa, expressed in the salivary glands of *An. sundaicus*. These proteins exhibited a strong reaction in 10-12 out of 20 serum samples in western blotting results of human sera. The reaction was highly specific and generated anti-salivary gland protein IgG antibodies; thus, these proteins could be developed as new biomarkers of exposure to malaria vectors or new candidates for multivalent malaria vaccines containing different component of the malaria transmission cycle. Therefore, further studies are needed to determine the identities of these proteins and their biological functions in malaria transmission and blood feeding using transcriptomic and proteomic analyses.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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