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Expression of PfHRP-2 Antigen in the Saliva of Malaria Patients

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A B S T R A C T

Malaria diagnosis is relatively easy, but making the blood film is still an obstacle in the field because it is invasive, requires expertise, and increases the risk of transmission of infectious diseases. Antigen detection of several microorganisms in saliva, the opportunity to make saliva as an alternative diagnostic specimen for malaria. This study examines the presence of malaria parasites PfHRP-2 antigen in saliva. Saliva was collected from falciparum malaria patients in some health centers in West Sumatra. PfHRP-2 antigen was examined by ELISA using a kit called CELISA antigen. PfHRP-2 antigen detection in saliva specimens was evaluated in the presence of *Plasmodium falciparum* on blood film as a gold standard. Out Of the 64 patients whose positive *Plasmodium falciparum* was confirmed by microscopy, 63 people tested positive for PfHRP-2 antigens by ELISA on blood specimens, and 32 were positive in saliva. PfHRP-2 antigens were not found in all the negative controls. Saliva can be used as a specimen for the detection of PfHRP-2 antigen. Development of detection methods necessary for the saliva specimens useful for malaria diagnosis.

Introduction

Malaria, a tropical disease caused by the Plasmodium parasite, is still a global health problem. An estimated 249 million people worldwide are infected with malaria and 608,000 of them die each year (WHO, 2022). In Indonesia, the disease is found throughout the archipelago. Among the five parasites that cause malaria, *Plasmodium falciparum* is the most virulent species because it can cause death (Akala, 2021).

Until now, the gold standard for the diagnosis of malaria is the discovery of Plasmodium in peripheral, thick and thin blood smears by microscopy examination. Although cheap, examination with a microscope requires adequate skills and experience, difficult to implement in remote areas, especially those without access to electricity. Whereas most malaria patients are in these remote areas (WHO, 2022). Since the discovery of the *Plasmodium falciparum* Histidine Rich Protein 2 (PfHRP-2) protein as a diagnostic

marker in *Plasmodium falciparum* and then the development of the use of rapid diagnostic tests (RDTs) based on the PfHRP-2 protein using anti-PfHRP-2 monoclonal antibodies (Das, 2017; Uyoga, 2021), the obstacles to malaria diagnosis in peripheral areas have begun to be overcome. RDT testing is generally relatively easy, fast, and accurate. Therefore, these tests do not require specialized laboratory personnel and can be performed anywhere, even in remote primary healthcare facilities without access to electricity (WHO, 2022).

PfHRP-2 is the main protein secreted by *P. falciparum* during the trophozoite and gametocyte cycle with the highest levels in mature schizonts, so the protein circulates in the peripheral blood of individuals infected with malaria parasites (Biswas, 2005; Amoah, 2016; Jang, 2018).

A large number of experiments have proven that PfHRP-2-based RDTs have high sensitivity and specificity in malaria-endemic and non-endemic areas, ranging from 96-100% (Jang, 2018).

Despite the convenience of RDTs, blood collection is still a bottleneck in the field as it is invasive, requires expertise, and carries the risk of contracting an infectious disease. These barriers are also found in the diagnosis of other diseases (Mharakua, 2006).

To overcome the obstacles associated with blood collection, researchers have come up with saliva as an alternative diagnostic specimen for several diseases, as saliva collection is painless, carries no risk of infection, and can be collected in relatively large quantities by anyone with little training (Chai, 2022).

Biomarkers that cross from the blood vessels into the salivary glands can in principle be detected in saliva (Sutherland, 2009). The diagnostic benefits of saliva have been demonstrated in several studies for several infectious diseases such as ebola virus (Formenty, 2006), measles, hepatitis, rubella (Nokes, 2001), and HIV (Kaufman, 2002).

Recently, biomarkers for malaria have also been reported to be identified in saliva (Wilson, 2008; Aninagyei, 2020). They have detected PfHRP-2 in the saliva of malaria patients using enzyme-linked immunosorbent assay (ELISA).

Indeed, in this study, the accuracy of the saliva test was still much lower when compared to blood or plasma. However, by paying attention to things that can affect the results of the examination in saliva, it is hoped that the sensitivity of the examination can be increased.

Many factors affect the results of the examination in saliva, including the level of the target protein to be examined, which is also influenced by the level of the protein in the blood (Sutherland, 2009), the collection method (Nunes, 2012), transportation/storage (Albertini, 2012) and variations in the gene that encodes the PfHRP-2 protein (Baker, 2005). Until now there has been no research on what level of PfHRP-2 in the blood will give a positive result in saliva.

With the right method, it is hoped that it will be able to increase PfHRP-2 levels in saliva so that later PfHRP-2-based RDT examinations which have only been in blood can also be applied to saliva specimens.

The purpose of this study is to prove that PfHRP-2 antigen can be detected in saliva specimens of malaria patients.

Materials and Methods

Sample Collection

For all patients suspected of malaria at the study site (Siberut Health Center working area during mass blood survey (MBS)), finger blood was taken for the preparation of thick and thin blood smears. Those who were positive for *P. falciparum* and met the inclusion criteria were asked to participate in the study and given written informed consent, for those under 18 years of age the informed consent was

signed by their parents. In *P. falciparum*-positive individuals, 5 cc of venous blood was drawn and asked to collect saliva into a sterile aseptic bottle until the volume reached 3-4 cc. The blood was placed into a vacutainer containing EDTA. Both types of specimens were then stored at -70 C until the ELISA test was conducted.

Thick and thin blood smears were made and stained with 10% Giemsa according to standard protocols. The examination was performed with a microscope using immersion oil with 1000 times magnification at 200 field of view by two experienced laboratory personnel. Malaria was declared positive if Plasmodium was found on thick/thin blood slides by both examiners and negative if no Plasmodium was found in 200 thick/thin field of view. For positive slides, species identification was done.

On positive slides, parasite density was calculated using the formula: The number of asexual stage *P. falciparum* found per 25 field of view on thin blood slides multiplied by 1000 or the number of asexual stage *P. falciparum* found per 200 leukocytes on thick blood slides multiplied by 40.

Blood and saliva were tested for PfHRP-2. For validity, it was compared with malaria-negative controls.

Examination of PfHRP-2 by ELISA technique

PfHRP-2 was tested using a commercial ELISA kit (Malaria Ag CELISA; Cellabs Pty. Ltd., Brookvale, New South Wales, Australia). This kit uses 2 different monoclonal antibodies to target different epitopes of the PfHRP-2 antigen. The assay is performed according to the manufacturer's instructions.

The plates were pre-coated with anti-*P. falciparum* IgM monoclonal antibody. To generate a standard curve of recombinant PfHRP-2, the recombinant antigen was serially diluted in 100 1 of 0.1 M with PBS containing 0.01% Tween 20 (PBS/T), doubled, starting from 1 5g/ml. The 10 g/ml standard stock was stored at 80°C. Then secondary antibody (100 l) conjugated with horseradish peroxidase was added, and the mixture was incubated at room temperature for 15 min.

The mixture was then transferred to the precoating plate and incubated at 37°C for 1 hour. The plate was washed 8 times with PBS/T that was available in the kit. The tetramethyl benzidine substrate provided in the kit was added and then incubated for 30 min at room temperature and darkness. The reaction was stopped by adding sulfuric acid. Results were read with a spectrophotometer (BioRad).

Research ethics

This study has received permission from the ethics commission of the Faculty of Medicine, UNAND.

Data Analysis

Data were presented in tabular form. Diagnostic performance was evaluated based on the results of ELISA's examination of blood, saliva, and peripheral blood smears.

Results and Discussion

Table 1 shows that of the 121 malaria-positive respondents examined using a microscope, 64 (52.9%) contained *P. falciparum* mono-infection, and the rest were P.vivax and mixed infection. No *P. malariae* or *P.ovale* parasites were found. The density of *P.falciparum* parasites ranged from 1200 - 19,600/ul blood. Thus the study subjects who met the inclusion criteria were 64 people.

The study subjects consisted of 42 females (65.6%) and 22 males (34.4%), ages ranging from 3 years to 75 years, three of whom (4.6%) were <10 years old, and 61 people (95.4%) were >10 years old.

The temperature at the time of sampling ranged from 36^0C-41^0C, where 40 people (62.5%) had no fever, and 24 people (37.5%) had a fever. None of

the patients had severe malaria (table 2). In theory, there is no gender difference in malaria incidence, but in this study, malaria parasites were found more in women. This can be explained because, at the time of this study, blood tests were often carried out on days coinciding with mothers' classes, at which time most pregnant women gathered, so that many were examined were women, while men were rarely at home during the day because they went to work or to the fields.

The examination conducted during the mass blood survey (MBS) was a peripheral blood examination of all residents found on that day regardless of whether there were malaria symptoms or not, and it turned out that most of those who contained malaria parasites at the time of the MBS were asymptomatic.

The presence of parasites in asymptomatic populations is often found in malaria-endemic areas. This indicates that the person concerned has gained immunity.

There are two types of immunity to malaria, namely immunity to infection and immunity to disease (Rochford, 2020). In this study, immunity to disease was found, although it is known that immunity to malaria is only partial and does not last long.

Table 1 shows that the density of malaria parasites in this study was low, which can be explained because in this study most respondents were residents who were not sick or did not show symptoms, in other words, they were people who contained malaria parasites, but their immunity had been able to suppress the number of parasites in small amounts so that they were below the clinical threshold.

To avoid false positives in controls, 30 control samples were obtained from people who did not have a history of malaria and lived in a non-malaria-endemic area, namely Padang City. In this study, none of the controls contained PfHRP-2 antigen. Table 3 shows that PfHRP-2 antigen was detected in 63 blood samples (98.4%). Only one sample did not detect PfHRP-2 antigen, although the microscope examination was positive. The temperature at the time of examination was 37.6 ^{0}C.

There was a time gap between finger blood examination and venous blood collection, it is likely that the person concerned had received malaria drugs before venous blood collection, but was unnoticed by the officer.

Qualitative detection of PfHRP-2 has become an alternative for malaria diagnosis in remote areas. It is an effective clinical indicator of past and current parasitemia. However, the use of PfHRP-2 in monitoring therapy response is still limited (Jang, 2018).

Table 4 shows that PfHRP2 antigen was detected in 32 saliva samples (50%). None of the control samples contained PfHRP2 antigen. When compared with previous studies, it is not much different from the results of Fung's research which obtained 47%.

There is growing interest in using saliva as an alternative diagnostic medium to replace blood because it is easier and reduces biohazards. PfHRP2 was selected as a target biomarker based on its characterization and previous use in commercial RDTs. PfHRP2 is secreted by the parasite at all stages, exported through the membrane of infected red blood cells, and then fully released into the blood at the time of schizont rupture (Fung, 2012).

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Table.1 Microscope examination results

Plasmodium type	Measure (%)	Parasite density
P. falciparum	64 (52,9%)	1200-19.600
P. vivax	37 (30,6%)	240– 5.500
P. malaria	-	-
P. ovale	-	-
Mix infection	20 (16,5%)	
Total	121 (100%)	

Table.2 Characteristics of Research Subjects N = 64

Characteristics		f	%
Sex	Male	22	35,4
	Female	42	65,6
Age	< 10 years	3	4,6
	≥ 10 years	61	95,4
temperature	< 37,5 ^{0}C	40	62,5
	≥ 37,5 ^{0}C	24	37,5

Table.3 Results of PfHRP-2 Antigen Screening in blood vs. microscopy examination in samples and controls

	PfHRP-2 antigen in blood		Total
Microscope Examination	+	-	
Malaria	63	1	64
control	0	30	30
Total	63	31	94

Table.4 Results of PfHRP-2 Antigen Examination in saliva vs Microscopy examination in samples and controls

	PfHRP-2 antigen in saliva		Total
Microscope examination	+	-	
Malaria	32	32	64
control	0	30	30
Total	32	52	94

The primary structure of PfHRP-2 contains many repetitive histidine sequences and is thought to present multiple epitopes for other antibodies (Baker, 2011). The degree of multivalence can vary with different PfHRP-2 levels and strains. While multivalence improves signal detection in serological examinations, interpretation is also complicated by the genetic diversity of the antigen. Reports from cross-reactions (Gamboa, 2010) indicate that some epitopes on PfHRP-2 are highly homologous to PfHRP-3. It is thus possible that the total antigen level includes other histidine-rich

proteins present in saliva. Finally, as discussed above, the saliva of semi-immune individuals can contain a mixture of free antigens and bound antibodies. Only the free fraction of PfHRP-2 will produce an ELISA signal.

Serum molecules can reach saliva through gingival sulcus fluid and intracellular and extracellular transport mechanisms. The transport of proteins into saliva depends on molecular mass, solubility, ionization (Gamboa, 2010), and salivary pH. Therefore, different molecules may undergo varying degrees of dilution during transfer from plasma to saliva. While the exact route followed by PfHRP-2 is not yet known, it most likely enters the salivary ducts by way of pericellular ultrafiltration from surrounding blood vessels. Further investigations that address the mechanism will be able to help optimize sample collection.

For analysis by ELISA, collection and stable storage of saliva samples are important to minimize sample degradation. At room temperature, salivary protein degradation occurs within 30 minutes after collection. In longer procedures, protein degradation can be reduced by processing at 4°C and adding protease inhibitors (Fung, 2012). In this study, as -80°C storage was not available in the field, all samples were stored at -20°C and used within 14 days thereafter. The various components of the saliva matrix can have a major impact on ELISA performance. A common approach to reducing the impact of the saliva matrix is to dilute the sample in a buffer and measure it against a calibration sample in the same buffer. Calibration standards are included on each microtitre plate to account for variation between plates.

PfHRP-2 antigen can be detected from saliva specimens of malaria patients

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