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Original Article

Low Allelic Variation of *Plasmodium falciparum msp-1 and msp-2* among Gold Miners in Central Kalimantan Province, Indonesia

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Abstract

Background: We aimed to find out the allelic variation of *Pfmsp-1* and *Pfmsp-2* among gold miners in Central Kalimantan Province, Indonesia using parasites' DNA isolated from archived RDT and GSBS

Methods: This study was done using the samples collected between 2017-2020 from health centers in Subdistrict of Mihing Raya, Danau Rawah, and Bukit Hindu as well as Kapuas District Health Laboratory in Central Kalimantan Province, Surabaya, Indonesia. Parasites DNA were isolated from RDT cartridges and GSBS of local and migrant gold miners. Species of *Plasmodium* were confirmed by single step PCR. The allelic variation of *Pfmsp-1* (K1, MAD20, RO33) and *Pfmsp-2* (3D7, FC27) were analyzed by nested PCR.

Results: *Pfmsp-1* gene was found in only two (22.22%) out of 9 local samples, and 3 (27.27%) out of 11 migrant samples were found positive for K1 (150 bp) as well as MAD 20 (190 bp) allelic families. *Pfmsp-2* gene were found in each one sample of 550 bp fragment in local (11.11%) and migrant samples (9.09%) for 3D7, and 2 samples of 300 bp fragments in local (22.22%) and 3 samples of 300 bp in migrant samples (27.27%). No difference in size and number of infections between both populations. The RO33 allelic family Alhamdulillah was not found in any sample.

Conclusion: Low allelic variation of *Pfmsp-1* and *Pfmsp-2* genes with monogenotype indicated the low intensity of malaria transmission among gold miners in the studied areas. Further, the transmission may occur locally in the mining sites.

Introduction

Malaria is a devastating disease that occurs mostly in tropical and subtropical countries around the world. Malaria is closely related to poverty and underdevelopment (1). Gold mining has played an important role in increasing national economic of mineral rich countries, but simultaneously can maintain the persistence of malaria in gold mining areas, especially illegal gold mining (2).

Malaria cases have been reported in gold mining areas in Indonesia (3, 4), French Guiana (5), Guyana, (6), and Colombia (7). In Indonesia, small-scale mining of the gold and coal, has increased since the onset of the economic crisis during about the year of 2000 (3). The new ponds ex illegal gold mining were left by the miners and became breeding places of *Anopheles* mosquito in West Kalimantan (8). Malaria patients were found in areas around illegal gold mines in South Kalimantan (9). The difficult access to the gold mines, which were located in the forests of the slopes of mountain contributed to the increased of malaria cases due to the increasing contact with *Anopheles* mosquito during their journey to and from gold mines (10). Gold miners in Central Kalimantan are five times more likely to be infected with malaria than non-miners (11). Malaria cases among gold miners in Kapuas and Gunung Mas Districts have been recorded by local health centers, however there was no formal publication.

Plasmodium falciparum merozoite surface protein-1 (*Pfmsp-1*) and *P. falciparum* merozoite surface protein-2 (*Pfmsp-2*) are the major protein that important for erythrocyte invasion (12), that can induce immune response in human (13), and are the promising candidate for an erythrocytic stage malaria vaccine (14). Three polymorphic allelic families in block two of *Pfmsp-1* gene has been identified as K1, MAD20, and RO33 (15). The *Pfmsp-2* has two allelic families in the central domain, and identified as 3D7 and FC27 (16). The genetic diversities of these two genes have been reported from different parts of the world (17, 18) to describe the population genetic structure of *P. falciparum* in the different malaria endemicity (19) as well as to distinguish treatment failure (20). Further, the genetic diversity of *P. falciparum* population is an important indicator of malaria transmission intensity in an area (21).

Parasite-based malaria diagnostic test either by microscopy examination or rapid diagnostic test (RDT) is recommended by the WHO for all patients suspected malaria before medication is provided (22). The used RDT and microscopy slides usually were then archived for years in the laboratory. Covid-19 pandemic has hampered malaria field survey such as active and passive case detection especially in collecting fresh blood from the patients for diagnosis, treatment, mapping as well as epidemiological and molecular studies. Alternatively, the archived RDT cartridges and microscopy slides of Giemsa-stained blood smears (GSBS) could be used as DNA sources for molecular study (23, 24). The RDT and GSBS have long been used to diagnose malaria among gold miners in Kapuas District.

Therefore, this study aimed to find out the allelic variation of *Pfmsp-1* and *Pfmsp-2* among gold miners in Kapuas District of Central Kalimantan Province, Indonesia using parasites' DNA isolated from archived RDT and GSBS.

Materials and Methods

Sample collection and ethics

This study was done during 2021 using the samples collected between 2017-2020 from health centers in Subdistrict of Mihing Raya, Danau Rawah, and Bukit Hindu as well as Kapuas District Health Laboratory in Central Kalimantan Province. The samples of positive *P. falciparum* and *P. vivax* those have been archived since the year of 2017 to 2020. The

collected RDT cartridges and microscopy slides have been used to diagnose malaria infection among gold miners who developed fever seeking medication to the health centers during that time. The origin of samples was further divided into local and migrant populations. Local population in the studied areas is called Dayak (25). Migrant population those came from various different ethnicities, from other cities, other provinces and even other islands who have lived in these districts for more than three months (9). The archived RDTs and GSBS microscopy slides were used as a source of DNA for molecular confirmation of *Plasmodium* species by single step PCR. Only DNA extracted from *P. falciparum* and mix infections were used for PCR-based genetic genotyping of *Pfmsp-1* and *Pfmsp-2* genes.

The research was done after receiving certificate of ethic for research from Health Research Ethics Committee, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia as stated on the certificate number 10/EC/KEPK/FKUA/2021 dated on January 18, 2021.

DNA extraction

The individual RDT cartridge was opened by using sterile scalpel and removed the nitrocellulose strip and stripped of any plastic covering the nitrocellulose (26). New scalpel was used for every single RDT cartridge. DNA extraction was done by using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following manufactures instruction.

The DNA from GSBS of microscopy slides was isolated by scratching off the smear and directly applied to the DNA isolation kit.

Species confirmation using single step PCR

The extracted DNA were then used as template for single step PCR amplification. Primers used for single step PCR based on the sequence of small sub unit ribosomal RNA (ssu-rRNA) of *P. falciparum* and *P. vivax*. Only three primers were used in this PCR. The first primer amplifies sequence common to rRNA of both *P. falciparum* and *P. vivax*. Second primer is specific for *P. vivax* and third primer is specific for *P. falciparum*. First and second primers together produced a 266 bp product specific for *P. vivax*, whereas first and third primers produced a 346 bp product specific for *P. falciparum*. The sequences of primers and PCR condition applied for this PCR were as described (27, 28).

PCR amplification and allele detection

PCR-based detection of allelic variation in Pfmsp-1 and Pfmsp-2 was done according to Aubouy method including primers and PCR condition with slight modification (13). The PCR products of single step PCR were used as template for nested PCR. The PCR products were then electrophoresed on 2% agarose gel. Determination of allelic variation of Pfmsp-1 was including K1, MAD20, and RO33, and Pfmsp-2 was including 3D7 and FC27 allelic families. The allelic variation was detected by visualization of the gel under UV illuminator, the sizes of bands were confirmed based on the 100 bp DNA ladder (Invitrogen).

Results

Characteristic of samples

Based on the data obtained from the health center of Kapuas and Gunung Mas Districts, a total of 101 samples consisted of 55 (54.46%) from local population further consisted of 30 (29.70%) of RDT cartridge and 25 (24.75%) of microscopy slides of GSBS, and 46 (45.54%) from migrant population, consisted of 29 (28.71%) RDT cartridges and 17 (16.83%) microscopy slides. There was no data of the origin of the migrants, however the information from the health centers noted that migrant community came from outside both districts, outside the province, and even outside Kalimantan (Borneo) Island. All samples in this study were collected from gold miners who sought medication due to malaria

infection during 2017-2020. All miners were male aged between 15-65 years old. The result of malaria diagnosis by RDT and microscopy examination are listed in Table 1.

Table 1: Malaria diagnosis by RDT and microscopy examination among gold miners originated from local and migrant population in Central Kalimantan Province during 2017-2020

	Number of sample (%)		
	LOCAL	MIGRANT	Total
P. falciparum	28(27.72)	23(22.77)	51(50.50)
P. vivax	27(26.73)	23(22.77)	50(49.50)
Total	55(54.45)	46(45.54)	101 (100)

Species identification by single step PCR

The purpose of this step was to confirm the species of *Plasmodium* based on DNA of parasites before further determination the allelic variation (Table 2). Twenty samples (19.80%) out of 101 samples were detected consisted of 7 samples (35%) of *P falciparum* (346 bp), and 13 samples (65%) of mix infection of *P. falciparum* and *P. vivax* (346 bp and 226 bp) as seen in Fig. 1. The samples consisted of 9 (45%) local, and 11 (55%) migrant population origins. These 20 samples were used for nested PCR genotyping.

Table 2: RDT and microscopy diagnosis of malaria compared with those of single step PCR

Spesies	***RDT and microscopy***		***Single-step PCR***			
	Pf	Pv	Pf	Pv	Mix	Neg
Pf	51		6			45
Pv		50	1	3	13	33
Total	101			101		

Pf: *P. falciparum*; Pv: *P. vivax*; Mix: mix infection of Pf and Pv; Neg: negative

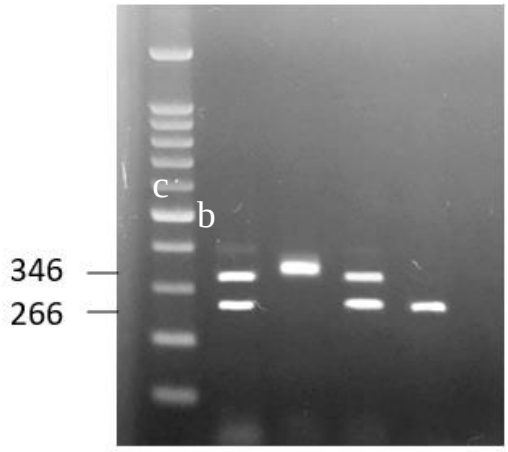

Fig. 1: Representative picture of single step PCR is showing single band of 346 bp for *P. falciparum* (a), 226 bp for *P. vivax* (b), and double band for mix infection of both species (c)

Allelic variation of Pfmsp-1 and Pfmsp-2

Pfmsp-1 gene was found in only two (22.22%) out of 9 local samples, and 3 (27.27%) out of 11 migrant samples were found positive for K1 (150 bp) as well as MAD 20 (190 bp) allelic families. *Pfmsp-2* gene were found in each one sample of 550 bp fragment in local (11.11%) and migrant samples (9.09%) for 3D7, and 2 samples of 300 bp fragments in local (22.22%) and 3 samples of 300 bp in migrant samples (27.27%). The RO33 allelic family Alhamdulillah was not found in any sample. The representative pictures of these allelic variation is shown in Fig. 2. Single band indicated a single clonal infection of *Plasmodium*. There was no different number of PCR fragments nor different size of the allelic family between indigenous and migrant population.

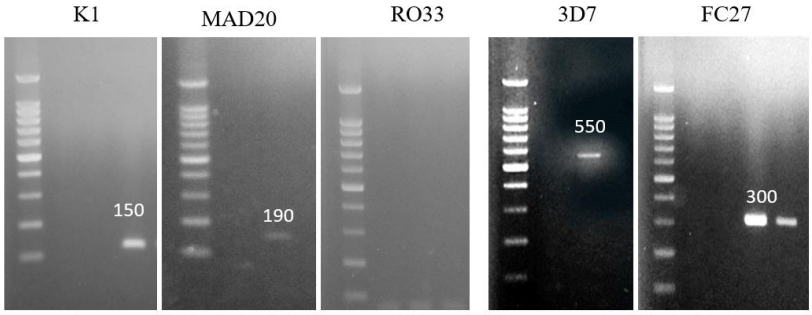

Fig. 2: Allelic variations of *Pmsp-1* and *Pfmsp-2* genes based on the DNA extracted from RDT cartridges and GSBS, are showing a 150 bp in K1, 190 bp in MAD20, and negative in RO33 of *Pfmsp-1* allelic family, a 550 bp of 3D7 and 300 bp of FC27 of *Pfmsp-2* allelic family

Discussion

Kalimantan (Borneo) island is one of the oldest artisanal and small-scale gold mining (ASGM) hotspot in Indonesia, where many people from outside the district even outside island areas came to work as illegal gold miners (4). The gold mining are often located in the forest or mountain slopes, where the miners have to walk along the areas where the contact with *Anopheles* mosquitoes were unavoidable without any kind of repellent to reach mining areas. This became an interesting field to study the genetic diversity of parasites among the malaria-infected gold miners. The results showed that the DNA could be extracted from RDT cartridges and GSBS samples archived during 2017-2020 at room temperature. Parasites were trapped in the nitrocellulose of RDT for long time period at room temperature. The results of PCR proved that DNA of parasites in this condition remained save for molecular study. Further, the older RDT that were collected during 2014-2017 as a source of DNA have been reported for sequencing and studying the molecular marker of anti-malaria resistance in República da Guiné-Bissau, West Africa (29). The RDT stored at 19°-25°C were also good tool for malaria diagnosis and also useful for molecular epidemiology and *Plasmodium* genetic analysis (30).

The differences in host geographic origins with different immune status might cause the differences in the host immune response to parasites which ultimately create diversity in parasite genetics (30), which will complicate the identification of *P. vivax* and *P. falciparum* (31, 32). The antigenic diversity of *P. falciparum* and *P. vivax* populations in an area plays an important role in the formation of natural immunity against malaria. The rapid and severe destruction of erythrocytes corresponds to the number of parasites found in the blood

by microscopic examination of the blood smear. The parasitemia is also influenced by the characteristics of the patient, such as age, sex, and race. The factors such as meiotic recombination events during the fertilization process in *Anopheles* mosquito might cause genetic variation of malaria parasite strains (18, 33).

The allelic variation of *Pfmsp-1* and *Pfmsp-2* based on the DNA isolated from archived RDT and GSBS, proved the DNA preparation by this method could be used in the genetic study. The results showed that amplified product from the Pfmsp-1, Pfmsp-2 genes were not varied in size, and there were low allelic variations between local and migrant gold miners. Besides, regarding the small number of samples, this condition might reflect the real condition of the circulating genes among the gold miners in the studied areas. Apparently, there was no new allelic variation were introduced by migrant gold miners. Malaria infection among migrant gold miners may occur in the location of gold mines. Therefore, the parasites that infect them probably are local parasites, and the genetic recombination in *Anopheles* mosquito occurred between local parasites, since the allelic variations were not different with those of local gold miners. This condition was also observed among *Pvmsp-1* and *Pvmsp-2* of *P. vivax*-infected patients in the same location (9).

Central Kalimantan Province is a malaria hypo endemic area, where the annual parasite incidence (API) is low in 2018 reached 0.24 (34). Only three districts of this province remained uncertified for malaria free (35). Low allelic variation of *Pfmsp-1* and *Pfmsp-2* genes with monogenotype indicated the low intensity of malaria transmission in the studied areas, since high rate of multigenotype infection indicated the high intensity of transmission(21). Genetic diversity of *Pfmsp-1* and *Pfmsp-2* in several districts in Indonesia are varies. Low genetic allelic variation found in this study is lower than that found in Lampung Province in Sumatra Island, where six variations in length range from 200 to 1000 bp of *Pfmsp-1* gene (36). Genetic diversity of *Pfmsp-1* in malaria hyper endemic area of Papua Province showed that MAD20 allelic family was predominant (20.8%), RO33 was 8.2%, and K1 was only 4.2% (37). Interestingly, the RO33 allelic family was not found in Central Kalimantan Province. This result is different with that found in Pahang, Malaysia, where RO33 allelic family was predominant as monomorphic (38), in contrast with that found in Grande Comoro Island where RO33 allelic family were poorly represented (2.4-4.0%) (39), while in Myanmar-China border was higher (12.56%) (17). In Gabon, when the DNA was isolated from RDTs showed that K1 (93.9%) was found higher than MAD20 (73.3%) (40). The allelic prevalence of FC27 in *Pfmsp-2* gene (15,38%) in this current study was higher than that of 3D7 allelic family (7,69%), similar to that found in Pakistan (41) and China-Myanmar border region (17). On the other hand in India, FC27 (12-23.3%) was found lower than 3D7 (35.7-56%) (18).

The various genetic polymorphism reported from various countries around the world proved that different population with different geographic conditions has a wide variety of different genetic variations.

Conclusion

The low allelic diversity of *Pfmsp-1* and *Pfmsp-2* genes among gold miners in studied areas indicated the low malaria transmission intensity. Further, the transmission may only occur locally between local and migrants' populations in the gold mining sites. Therefore, gold mining plays an important role in malaria transmission, and plays a barrier to malaria elimination in these areas. The specific malaria control program must be implemented in these areas to control the transmission and to avoid the spread of malaria to other regions.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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