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Baseline Parasite Profile in Developing Irradiation Malaria Vaccine in Indonesia: Molecular Analysis of Papua Samples

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Introduction: Malaria is still a major problem in Indonesia. Therefore the development of its vaccine that can be created with radiation is urgently needed. This research aims to determine the genotype of blood specimens obtained from 4–22 years old malaria outpatients in Jayapura General Hospital, Papua. The information obtained will be a great value in additional knowledge for vaccine development of irradiated gamma-ray parasites. *Methodology*: Microscopic examination on thin blood smear was done, followed by Polymerase Chain Reaction (PCR) diagnosis to further confirm the parasite using 18S rRNA gene and mitochondrial primers on deoxyribonucleic acid extracted using chelex-100 ion exchanger and the presence of each mutation in the genes was examined using restriction fragment length polymorphism method. *Results*: Our result showed that nine samples infected with Plasmodium falciparum and 1 with *P. vivax*. Beside that nine samples carried mutations in *pfcrt* and 22% in *pfmdr1*. Mutations involving N1042D in *pfmdr1* gene was also detected in 3 samples (33%). Mutations in *dhfr* gene at codon S108N were detected in all nine samples. Mutations in this gene at codon K540E have also detected in 3 (33%) samples. *Conclusion*: Analysis of the parasite genotype indicated that infection occurred with multiple parasite strains. The majority of the samples carried the 3D7 type of MSP2 gene while others carried the FC27 type. The MSP1 genes observed carried K1, MAD20, and RO33 types but some other types could not be determined.

Keywords: Genotyping, Malaria, Microscopic, PCR, RFL.

1. INTRODUCTION

Malaria continues to be one of the foremost causes of death in the developing countries such as Indonesia. In this country, around 15 million cases and 42,000 deaths were found every year.[1–3] The anti-malarial drug only partially effective in controlling disease due to the increasing problem of drug-resistant parasites all over the world. Thus, additional approaches such as vaccination have become a top public health priority for malaria control.[4]

Throughout Papua, Indonesia, malaria has been an important disease for many years, accounting for 16% of all hospitalizations, 14% of all hospital deaths, and 20% of all outpatient consultations.[5,6] Consequently, most febrile patients are considered to have malaria and are given empirical treatment out of fear of missing life-threatening *P. falciparum* infection.[7] Several factors may promote the development of antibiotic and antimalarial resistance and unnecessary morbidity and mortality. The first chloroquine-resistant isolates of *P. vivax* were reported from Papua in 1989.[8,9] This region currently has the highest rates of drug-resistant *P. vivax* in the world.[10]

Ionizing radiation has been successfully used to attenuate parasites and micro-organisms for vaccine development and research.[11–14] Irradiated pathogens frequently lost their reproductive ability or virulence, but retain the metabolic activities and morphology, and can stimulate a specific immune response. In some cases, the radio-attenuated pathogens are more immunogenic than the normal counterparts.[15]

Several *P. falciparum* genes show extensive genetic polymorphism. This phenomenon is exploited for genetic fingerprinting and for assessing parasite population dynamics. High polymorphism has been shown in msp1, msp2 and glurp genes in different geographical locations in malaria endemic areas.[16–21] Therefore, comparing the genotypes of these three loci at baseline and at the time of parasite recurrence would be expected to discriminate between recrudescent and new infections.[22,23] Comparing

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molecular genotypic pattern of pre-treatment (baseline) and recurrent infections provides a means to help characterize the recurrent parasites as a recrudescence, i.e., a true failure, or a new infection (either from pre-existing liver infection or a newly established infection from an infected mosquito bite), i.e., a successful treatment.

It already known that a malaria vaccine would be a valuable tool that offers the potential to reduce both the morbidity and mortality of the disease. The parasite has a huge repertoire of possible antigenic variants, this together with the ability to undergo rapid antigenic switching presents an important challenge to vaccine development. It has been over 30 years since the first successful malaria vaccine was trialed.[24] As it consisted of the bites of a thousand irradiated mosquitoes infected with the parasite. The feasibility of this approach option for large-scale use has been until recently dismissed. Further elucidation of the complex geographical patterns of *P. vivax* and *P. falciparum* variation will be important both for diversity assessments of genes encoding candidate vaccine antigens and in the formulation of control and surveillance measures aimed at malaria elimination.

Our results confirmed the propriety of this system for large-scale genetic analysis of *P. falciparum* which will provide innovative insight into the complex genetic structure of the malaria parasite and hugely accelerate efforts to develop novel intervention strategies such as a vaccine that can be created from attenuated parasites with ionizing radiation.

2. METHODS

2.1. Subjects

This study is part of a experimental research on the development irradiated sporozoites as malaria vaccine materials that took place in Jayapura, Papua Province, Indonesia with quite highly malaria transmission intensities. For all the studies ethical approval was obtained from Ethics Committees of National Institute of Health Research and Development, Ministry of Health. Informed consent form was signed by all subjects who suspected infected with malaria parasites as they were as outpatient in Jayapura General Hospital. And blood samples from participant were collected in 5.0 ml BD Vacutainer. In this research, 10 blood samples were studied.

2.2. Microscopic Examinations

Thick blood smears were prepared for all blood samples and stained with 10% Giemsa. The malaria parasite was detected under light microscopy using 1,000× oil immersion by an experienced microscopists from the Hospital. At least 200 ocular fields were examined before considering a slide negative, and parasite densities were counted as parasites per 200 leukocytes and reported as parasites/mm^3 assuming a white blood cell count of 8,000/mm^3.[25]

2.3. DNA Extraction

Parasite and human host DNA was obtained from blood samples using chelex-100 ion exchange (Biorad Laboratories, Hercules, CA, USA) according to a procedure described previously.[26] The DNA was either used immediately for PCR amplification or stored at −20 °C for later analysis.

2.4. Molecular Genotyping

PCR amplification of template DNA and analysis of region II of glurp, central polymorphic region of msp2 (3D7 and FC27 allelic families), and block 2 of msp1 (K1, MAD20, and RO33 allelic families) was performed in accordance to the recently recommended genotyping protocol[27] with minor modification. The primary and nested PCR amplification of the msp1 and glurp loci were carried out at Malaria Laboratory of Eijkman Institute, and the conditions were as described elsewhere.[28] The DNA amplified was analyzed by electrophoresis on 2% agarose gel and stained in 0.5 ug/ml of ethidium bromide solution, and the band was visualized under UV light.

2.5. Molecular Resistance Examination

To identify the presence of point mutations in molecular markers of *P. falciparum* drug resistance, we performed a series of PCRs with specific primers that amplify informative regions of the *P. falciparum* multidrug resistance 1 (*pfmdr1*), *P. falciparum* chloroquine (CQ) resistance transporter (*pfcrt*), and dihydrofolate reductase (*dhfr*) genes. Part of the *pfcrt* gene containing the first predicted transmembrane domain was amplified as described.[13, 28] The PCR products were analyzed for the Lys76Thr mutation by restriction fragment length polymorphism using *Apo*I (New England Biolabs, Beverly, MA). Restricted amplification products were subjected to electrophoresis on 10% polyacrylamide gels and visualized by staining with ethidium bromide. The regions of the *dhfr* and *dhps* gene containing the mutations involved in anti folate resistance were also amplified as described.[28] Mutations in the *dhfr* gene at codon Ser108Thr/Asn were identified with mutation-specific primers. Mutations in the *dhfr* gene at codons Ala16Val, Asn51Ile, Cys59Arg, and Ile164Leu and in the *dhps* gene at codons Ser436Phe, Ala437Gly, Lys540Glu, Ala581Gly, and Ala613Ser were also determined by a nested PCR amplification followed by size fractionation on agarose gels.[28]

3. RESULT

3.1. Microscopic Observation

Microscopic and molecular examinations followed by restriction test were subjected to all samples according to the standard procedures. Results showed that microscopically all blood smears were infected with malaria parasites, of which one was infected with *P. vivax* (Fig. 1). This was confirmed by polymerase chain reaction (PCR) that revealed that nine subjects had *P. falciparum* infection and one was *P. vivax* malaria.

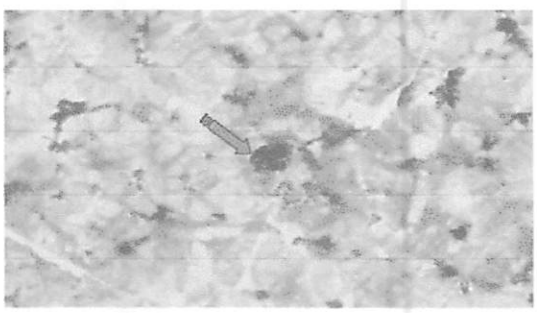

Fig. 1. Giemsa stained thick blood smear showing *P. vivax* parasite infection in one sample found in this research (arrow).

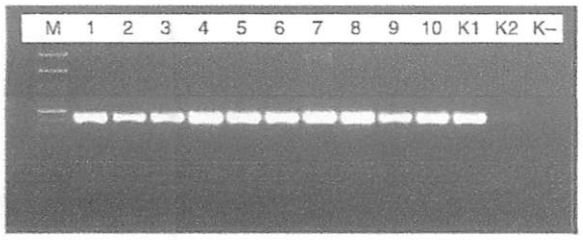

Fig. 2. PCR results in DNA mitochondrial gene where all are positive of *Plasmodium sp.*

3.2. Species Identification

PCR analysis using mitochondrial gene found that all samples were positive for *Plasmodium sp* (Fig. 2). However, genotyping using *Glurp* primer revealed that one sample was negative (Fig. 3). Identification of species with restriction fragment length polymorphism (RFLP) using Ssp1 enzyme showed that nine were positive for Pf and 1 sample was *P. vivax* infection, whereas with AclI enzyme 1 sample was Pv and nine samples were Pf. A total of 9 *msp1* genotypes (three MAD20-, two RO33- and four K1-types); 9 *msp2* genotypes (7 3D7 and 2 FC27-types) and nine glurp genotypes were recorded (Table I). From these, it can be known that all samples were positive for malaria with mostly were *P. falciparum* infections and the development of vaccine should be focused on this species. *Msp-1* and *msp-2* are highly polymorphic single copy genes and have been employed to investigate parasite genetic diversity all around the world, and also are under extreme immune selection pressure.

Thick-section microscopy of blood-stage *P. vivax* parasites infected sample from study area is presented in Figure 3. There is no trophozoite, ring and schizont parasites forms are shown this figure. However, it was recognized based on its specific characteristics of thick blood smear.

3.3. SNP Study

Further study in the 16 single nucleotide polymorphisms covering *pfcrt*, *Pfmdr1*, *dhfr* and *dhps* genes on these ten samples revealed that two out of 9 samples analyzed had a polymorphism. The complete results are presented in Table II. These are found in sample no. 7 and eight where polymorphism is N86Y.

Here *Afl*III restriction enzyme was used to analyze and detect mutation point at codon 86 (N86Y) and *Dde*I for mutation point at codon 1034 (S1034). Amplification of *pfmdr*1 generated

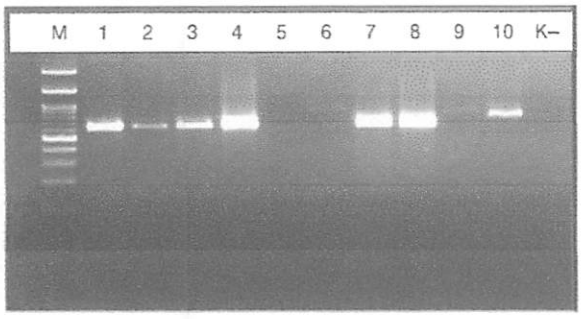

Fig. 3. Genotyping of the samples using Glurp primers where one sample was negative (sample no. 5).

Table I. Positive results in MSP1, MSP2, and Glurp from 10 samples analyzed.

	MSP1			MSP2		
No. sample	MAD 20	K1	RO33	3D7	FC27	Glurp
1	–	+	–	+	–	+
2	–	–	–	+	–	+
3	–	+	–	+	–	+
4	–	+	–	+	–	+
5	–	–	–	–	–	–
6	+	–	–	–	+	+
7	–	–	+	+	–	+
8	–	–	+		–	+
9	+	+	–	+	+	+
10	+	–	–	+	–	+

372-bp PCR product. Restriction of the fragment using *Afl*III produced 248-bp and 124-bp in the mutation of N to Y at position 86. Whereas, the amplification of *pfmdr1* using 1034-F and 1034-R (Table I) generated 189-bp PCR product (Table II). Three polymorphisms were lack to be analyzed due to the fail in enzymatic cleavage that was due to un appropriate condition of PCR and enzymatic process.

In this research nine samples carried mutations in *pfcrt* (all sample showed nucleotide alteration from K to T) and 5 of 9 (22%) sample had mutation in *pfmdr1* (2 of them were in N86Y). Mutations involving N1042D in *pfmdr1* gene was also detected in 3 samples (33%). Mutations in this gene at codon K540E were also detected in 3 (33%) samples (no. 6, 7 and 8). No results were obtained for S1034C mutation of *Pfmdr-1* and A16V of *dhfr* genes as well as in Atp-6 due to technical problem.

More studies are warranted to examine the same thing with much larger number of samples to get a more comprehensive pattern in genotyping the parasites for development of irradiation vaccine for protective immunity.

Table II. Detailed SNP results of each genes representing their nucleotide alterations on ten samples of parasited infected blood obtained from Papua, Indonesia. Ten samples were studied for 15 SNP's of *P. falciparum*.

	No. of samples and its nucleotide alterations									
Poly-morphism	1	2	3	4	5	6	7	8	9	10
Pfcrt gene										
K76T	T	T	T	T	–	T	T	T	T	T
Pfmdr-1 gene										
N86Y	N	N	N	N	–	N	Y	Y	N	N
S1034C	ND	ND	ND	ND	–	ND	ND	ND	ND	ND
N1042D	N	N	N	N	–	N	D	D	D	N
D1246Y	D	D	D	D	–	D	D	D	D	D
dhfr gene										
S108N	N	N	N	N	–	N	N	N	N	N
N51I	N	N	N	N	–	N	N	N	N	N
C59R	C	C	C	C	–	C	C	C	C	C
A16V	ND	ND	ND	ND	–	ND	ND	ND	ND	ND
dhps gene										
A437G	A	A	A	A	–	A	A	A	A	A
K540E	K	K	K	K	–	E	E	E	K	K
S436A	S	S	S	S	–	S	S	S	S	S
G581A	G	G	G	G	–	G	G	G	G	G
A581G	G	G	G	G	–	G	G	G	G	G
A613S/T	S/T	S/T	S/T	S/T	–	S/T	S/T	S/T	S/T	S/T
Atp-6	ND	ND	ND	ND	–	ND	ND	ND	ND	ND

Notes: Sample no. 5 as *P. vivax* infected blood was not determined, ND: no data available due to the lack of enzymatic cleavage.

4. DISCUSSION

Malaria poses a serious public health problem in many parts of the world and approximately half of the world's population is at risk, in particular those living in lower-income countries.[29] According to US Global Health Policy Reported Malaria Deaths 2010. Indonesia ranks 28 of countries with highest case of malaria (432 cases/100.000).[30] It is by the fact that malaria remains a major public health problem in Indonesia, with 30 million cases and 120,000 deaths annually. Recently measures of annual parasite incidence (API) have varied substantially between provinces, but the highest API is consistently detected in the eastern parts of Indonesia. Moreover, the control of malaria in Indonesia is still problematic due to lack of access to health facilities in very remote areas and hard to reach islands, inequality of health development, and limitation of adequate malaria human resources mostly microscopist and entomologist as well as genotypic.[31]

Antibodies to polymorphic antigens expressed during the parasites at erythrocytic stages are important mediators of protective immunity against *P. falciparum* malaria. Therefore, polymorphic blood stage antigens such as MSP3, EBA-175 and GLURP and variant surface antigens PfEMP1 and RIFIN are considered vaccine candidates. Molecular genotyping of highly polymorphic regions of *P. falciparum* msp1, msp2 and glurp loci is usually carried out to distinguish recrudescence (true failures) from new infections.[32,33] This tool has now been adopted as an integral part of anti-malarial and vaccine efficacy studies and clinical trials. However, there are concerns over its utility and reliability because conclusions drawn from molecular typing depend on the genetic profile of the respective parasite populations, but this profile is not systematically documented in most endemic areas of Indonesia to support the development of malaria vaccine with irradiation technique.[5,34]

We describe an analysis of the genetic variation of malaria parasites in a very limited number of samples compared to the high number of incidence that commonly found in Jayapura. Large-scale number should be conducted to maximise the statistical power and to avoid a little information on the development of vaccine for this disease created by ionizing radiation. Here we selected two polymorphic markers, the genes for merozoite surface protein 1 (MSP1) and MSP2, to genotype the *P. falciparum* isolates responsible for the infection. PCR amplification of these genes points out the presence of length polymorphism, allowing the detection of multiple infections by different *P. falciparum* genotypes.[35]

Genotype diversity existed among *P. falciparum* parasite in several countries such Colombia, China and Suriname. Villa et al.[36] from Colombia found wild-type form in codons 16, 59 and 164 of *dhfr* gene, while mutant found in codons 51 and 108. In *dhps* gene, the mutant 437 glycine was detected in 85% sample, while codons 436, 540, 581 and 613 were wild-type. While a study in China by Huang et al.[37] on total of 108 blood samples found that almost all (95.3%) parasites still carried the *pfcrt* K76T mutation, whereas the majority of isolates displayed the wild-type pfmdr1 N86 and D1246 sequences. The most prevalent mutation was pfdhfr C59R (95.9%). A437G ($n = 77$) and K540E ($n = 71$) were the most prevalent mutations in pfdhps, and 52.7% of the samples were double mutants, among which A437G/K540E was the most common double mutation (37/49). Quadruple mutants were found in 28.0% (26/93) of the samples. A total of 8.6% of isolates (8/93) carried the S436A/A437G/A581G triple mutation. Other study in Suriname on 86 patients with symptomatic malaria had been done. In this country mutations in the *pfcrt*, *dhps*, and *dhfr* genes were found in all samples tested, suggesting that resistance to chloroquine and antifolate drugs is present at a high frequency. A low number of alleles was found for the *msp-2* and *glurp* genes.[38]

5. CONCLUSION

In conclusion, our analysis showed that mostly blood samples were infected with *P. falciparum*. The majority of the samples carried the 3D7 type of *MSP2* gene while others carried the FC27 type. The *MSP1* genes observed carried K1, MAD20 and RO33 types but some other types could not be determined.

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