Genetic Diversity of Plasmodium falciparum

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Genetic Diversity of *Plasmodium falciparum* Glutamate Rich Protein in Patients Attending the Merauke Hospital in Papua Province, Indonesia

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ABSTRACT

Malaria remains an important health problem in Indonesia with the highest transmission in Papua Province, an eastern part of this country. The genetic diversity of malaria parasites is the main problem in understanding several aspects of malaria infections and the dynamics of their transmission, which also play a role in the development of a vaccine. *Plasmodium falciparum* is the deadliest of the human malaria parasites. *Plasmodium falciparum* glutamate-rich protein (Pfglurp) is one of the many erythrocytic stages antigens currently under development for a vaccine. The Pfglurp gene has been extensively used as a marker to investigate the genetic diversity, Multiplicity of Infection (MOI), the level of malaria transmission, immunity against malaria, as well as a discriminatory instrument to distinguish new from recrudescent infections of the field parasite population. Thus, this genotyping study aimed to find out the genetic population of *P.falciparum* at the Merauke District, Province of Papua, Indonesia. DNA samples were isolated from Dried Blood Spots (DBS) obtained from *P.falciparum* infected patients in the Regional Public Hospital of Merauke, Province of Papua, Indonesia during May 2019-July 2019. The isolated DNAs were then amplified for nested Polymerase Chain Reaction (PCR) prior to Pfglurp genotyping. The glurp gene was identified in all 51 DBS samples of *P.falciparum*-infected patients, and 18 variants of allele were found. Among them, 45.10% were found to bear multigenotype infections. The size of the dominant allele (12.5%) was 701-750 bp. The MOI was 1.58. The genetic population of *P.falciparum* in Merauke Hospital has contained a higher percentage of multigenotypes compared with monogenotypes indicating the high transmission of malaria in the studied area.

Keywords: *Plasmodium falciparum*, Pfglurp, genotyping, polymerase chain reaction

Malaria remains an important health problem in Indonesia. Papua province has the highest burden of malaria in Indonesia. Merauke is a district easternmost in Indonesia and still has the problem of malaria, the district orders directly with the state of Papua New Guinea. Annual Parasite Incidence (API) in the district Merauke in 2010 with 19/1000 inhabitants.[1] Although all Plasmodium species can be found in Papua, including *Plasmodium knowlesi* formerly found in Kalimantan, the most common type of infection in Papua is by *Plasmodium falciparum* and *Plasmodium vivax*. However, *P.falciparum* is the main cause of morbidity and mortality of malaria in Papua.[2,3]

Genetic diversity of *P.falciparum* is the major characteristic and a factor by, which the parasites survive the immune response of hosts. It results from allelic polymorphism, recombination, chromosome rearrangements, and antigenic variation.[4] Genetic diversity of malaria parasites represents a major issue in understanding several aspects of malaria infection and disease transmission dynamics and has hampered the malaria vaccine development.[5] The genetic diversity was influenced by several factors such as the irregular use of anti-malaria drugs, which cause parasite resistance to the drugs, people mobilization from malaria-endemic area to non-endemic ones.[6]

Plasmodium falciparum glutamate-rich protein (Pfglurp) is a protein expressed on both pre-erythrocytic and erythrocytic stages of the parasite as well as newly emerging merozoites has high immunogenicity and acts as an antibody target involved in cellular inhibitor in monocytes, has been extensively studied as a vaccine candidate.[7-9] The Pfglurp gene has been extensively used as a marker to investigate the genetic diversity, Multiplicity of Infection (MOI), the level of malaria transmission, immunity against malaria, as well as a discriminatory instrument to distinguish new from recrudescent

infections of the field parasite population.[10]

The genotyping of Pfglurp in the Merauke district, Papua Province has not yet been reported. This study was aimed to evaluate the genetic population of *P.falciparum* by nested PCR among malaria-infected patients attending the Merauke Hospital in Papua Province, Indonesia.

METHODS

Ethical clearance was obtained from the Ethics Committee for Health Research, Faculty of Medicine, Airlangga University with number 169/EC/KEPK/FKUA/2019. Dried Blood Spots (DBS) were acquired from patients suffering from malaria who visited the Emergency Department, Specialist Outpatient Clinic, and Inpatient Department of the Regional Public Hospital of Merauke, Papua during May-July 2019. The DNA isolation was performed according to the protocols of Norgen's Dried Blood Spot DNA Isolation Kit (Norgen, Thorold, Canada). The DNA was then stored at -2^0 C until used.

Two kinds of nested PCR were performed to identify Plasmodium species and to identify Pfglurp genotype variants. The PCR to genotype the Pfglurp used primers specific for glurp gene locus as shown in Table 1.[11]

The first PCR mixture consisted of 12.5 µL of 2x Go Taq green master mix (Promega, Madison, USA.), 2.5 µL of each primer forward and reverse (10 µM) and 1 µL of DNA template. The total volume was 25 µL. The PCR condition for glurp genotype can be seen in Table 2. Electrophoresis of PCR products was performed in a 2% agarose gel along with the DNA marker of 100 bp interval (Promega, Madison, USA). The DNA products were then visualized and documented by GeldocEZ imager (Biorad, USA). Furthermore, MOI was determined by dividing the total number of fragments by the number of samples positive containing glurp.[10]

The data were descriptively analyzed by counting the number of DNA fragments. The size of fragments was then differentiated every 50 bp of band sizes to identify the number of allelic variants and their frequency distribution. The allelic frequency was calculated by dividing the number of a particular allele by the total number of samples positive for that allelic family of the gene.[7] Multiplicity of infection was defined as the number of parasite genotypes per infection, which was obtained by dividing the total number of fragments by the number of samples positive containing glurp.[7,9,10,12,13] Twenty samples with more than one allelic variant were considered as a multigenotype infection while the presence of a single allelic variant was considered as monogenotype infection.[12]

RESULT AND DISCUSSIONS

The age of patients was grouped based on Depkes RI 2009 to categorize the genotype based on age group. The grouping of age can be seen in Table 3.[14]

Molecular diagnosis by nested PCR resulted in 51 samples, which were positive *P.falciparum* consisting of 34 (66.7%) males and 17 (33.3%) females. The

Table 1. Primer sequences for genotyping of Pfglurp

Amplification	Primer	Sequence (5'→ 3')
Primary	GF3	ACATGCAAGTGTGATCCTGAA
	GF4	TGTAGGTACCACGGGTTCTTGTGG
Secondary	GF4	TGTAGGTACCACGGGTTCTTGTGG
	GNF	TGTTCACACTGAACAATTAGATTTAGATCA

Table 2. The PCR condition for glurp genotype

PCR Step	Nested 1			Nested 2		
	T (^{0}C)	Time (minutes)	Number of Cycle	T (^{0}C)	Time (minutes)	Number of Cycle
Initial denaturation	95	5	1	94	5	1
Denaturation	94	1	30	94	1	35
Annealing	58	2		59	2	
Extension	72	2		72	2	
Final extension	72	10		72	10	1

T: Temperature

average age of patients was 10.08 years old. The representative picture of PCR-based diagnosis can be seen in Figure 1.

Genotyping by nested PCR resulted in all 51 *P.falciparum*-infected samples containing the glurp gene, and the second nest PCR showed the allelic variation of glurp genotype with the size of bands ranging from 250 bp–1315 bp. The representative picture of PCR-based glurp genotyping can be seen in Figure 2. Eighteen variants of the Pfglurp gene were found consisted of the PCR fragments with sizes ranging from 201 to 1250 bp. Correlation of the variants of Pfglurp genotype with a group of age showed that age group of 17-25 years old (late teen) contained the highest number of variants (19 variants) and the lowest number found in 0-5 years old age group (toddler), which contained 3 variants. The increased age of the number of variants tented to increase, however, decreased in 56-65 years old of age (late elderly) as shown in Table 4.

The Pfglurp gene locus has plenty of allele variations, enabling this genotyping to identify *P.falciparum* genetic variation in a population.[15] The Pfglurp gene allele variation is directly associated with endemicity and the number of evaluated samples.[16] The glurp genotype variation found in this study ranged from 250 bp to 1315 bp in size similar to in Pesawaran District, Province of Lampung, Indonesia, where 200-1200 bp were found.[17] A different result was inferred from a study in Central Sumba, East Nusa Tenggara, with a glurp gene of 700-1100 bp, and a study in Sulawesi and Kalimantan identified 580-119 bp.[18,19] The finding of 18 variants of the allele in this current study was the same as found in West Cambodia, while in southwestern Nigeria was 12, in moderate malaria-endemic areas in East Malaysia showed 5 allele variants.[12,20,21] This finding proved the different locations have a different population of parasite genetics as well. These diversities indicated the level of malaria endemicity worldwide. The three dominant allele variants were 701-750 bp, 651-700 bp, and 951-100 bp, respectively, which showed more than a 10% frequency (Figure 3). These allelic variant profiles are valuable baseline data for continued monitoring of polymorphisms associated with antimalarial drug resistance in these areas.

Based on the number of genotypes in glurp gene locus analysis resulted in the MOI of 1.57. The number of Pfglurp variant genotypes per 51 *P.falciparum*-infected samples found in the Merauke Hospital was 80. The MOI rate usually reflects the rate of transmission intensity, although its correlation is not linear.[22] The low MOI in the isolates with a higher parasite density shows the presence of dominant monogenotype infection without significant rivalry.[5] The monogenotype infection found in this study was 54.90% and multigenotype infection was 45.10%, as shown in Table 5. A monogenotype infection is an infection caused by a single genotype, whereas a multigenotype infection is caused by two or more genotypes in one sample within the same locus.[18] The multigenotype infection found in this study was higher than that was found in Thailand-Myanmar

Table 3. Grouping of the age of *P.falciparum*-infected patients at the Regional Public Merauke Hospital

Category	Age (years)
Toddler	0–5
Children	6–11
Early teens	12–16
Late teens	17–25
Young adults	26–35
Adults	36–45
Early elderly	46–55
Late elderly	56–65

Figure 1. Representative picture of *P.falciparum* species M: marker. The positive *P.falciparum* samples were at 206 bp (white arrow)

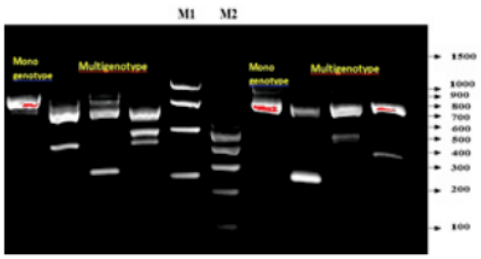

Figure 2. Representative picture of PCR-based Pfglurp genotyping. Genotype containing one PCR fragment is monogenotype and two or more fragments are multigenotype

Table 4. The frequency of genotype based on age group

Variant Allele	Size (bp)	Age (years)								Frequency (%)
		0-5	6-11	12-16	17-25	26-35	36-45	46-55	56-65	
1	201-250	-	1	-	1	-	2	1	1	6 ((7.5)
2	251-300	-	-	-	-	1	-	1	-	2 (2.5)
3	351-400	-	-	-	1	-	-	-	-	1 (1.25)
4	401-450	-	1	1	2	2	-	1	-	7 (8.75)
5	451-500	-	-	1	1	-	-	-	-	2 (2.5)
6	501-550	-	-	-	1	-	-	-	1	2 (2.5)
7	601-650	-	-	-	-	-	-	1	-	1 (1.25)
8	651-700	-	-	2	2	2	-	2	1	9(11.25)
9	701-750	-	1	-	4	1	1	2	1	10 (12.5)
10	751-800	1	-	2		1	1	-	2	7 (8.75)
11	801-850	-	-	-	1	1	2	-	-	4 (5)
12	851-900	-	-	3	1	1	1	1	-	7 (8.75)
13	901-950	1	-	-	1	-	1	1	-	4 (5)
14	951-1000	-	2	1	2	1	1	-	1	8 (10)
15	1001-1050	-	-	1	-	-	-	-	-	1 (1.25)
16	1050-1100	1	-	-	-	1	-	-	-	2 (2.5)
17	1151-1200	-	-	2	1	-	1	2	-	6 (7.5)
18	1301-1350	-	-	-	1	-	-	-	-	1 (1.25)
Number (%)		3 (3.75)	5 (6.25)	13 (16.25)	19 (23.75)	11(13. 75)	10 (12.5)	12 (15)	7 (8.75)	80 (100)

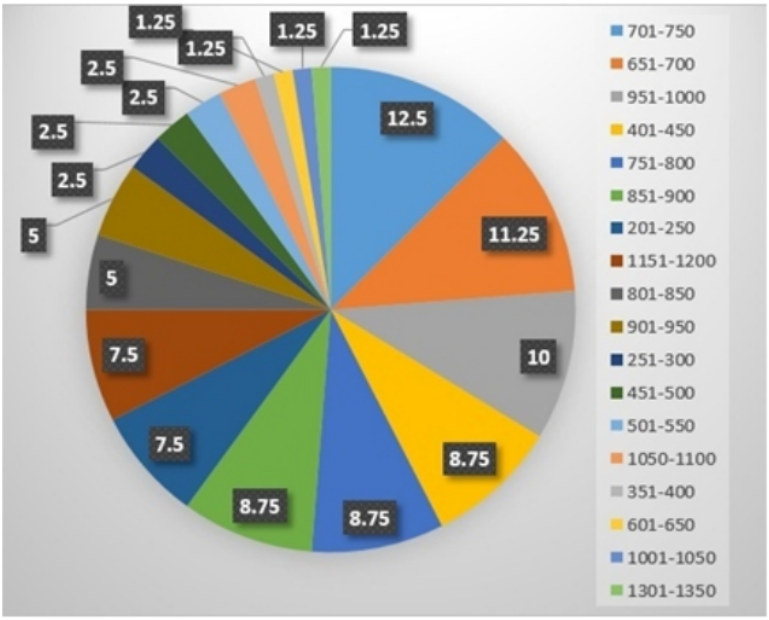

Figure 3. Pie chart of the frequency distribution (%) of Pfglurp gene in *P.falciparum* patients at the Regional Public Hospital of Merauke. The three dominant sizes of allelic variant genotype were 701-750 bp (12.5%), 651-700 bp (11.25%), and 951-1000 bp (10%)

Table 5. Summary of glurp genotypes in *P.falciparum*-infected patients attending the Merauke Hospital, Papua

Kind of Genotypes	Number of Samples (%)	Number of Genotypes (%)
Monogenotype	28 (54.90)	28 (35)
Multigenotype		
2 Genotype	18 (35.30)	36 (45)
3 Genotype	4 (7.84)	12 (15)
4 Genotype	1 (1.96)	4 (5)
Total number of multigenotype	23 (45.10)	52 (65)
Total number of genotype	51 (100)	80 (100)
MOI = 1.57		

border, which was 15.3%, while in Western Cambodia the multigenotype infection was 8%.[21,23] The high rate of multigenotype infection found in this study showed that the intensity of transmission at the study site was still high.[13] A study in Gabon that evaluated the correlation between multigenotype infection and cytoadherence (the adherence of parasite-infected erythrocytes) showed that multigenotype infection, as it turned out, had no significant effect on cytoadherence or the severity of malaria. The severity of malaria was also not affected by the type of certain genotype and the amount of infecting *P.falciparum* genotype.[24]

The use of glurp gene locus has its benefits in epidemiology tracking and to evaluate the transmission rate and the correlation of its factors to the immune system. An immunology study in a high and low malaria contagious area showed a high prevalence of glurp antibodies in adults, as well as a significant correlation between a specific high level of glurp antibody with low parasite density and clinical protection against malaria. Furthermore, *P.falciparum* infection often triggers antibody production as a response to glurp, which naturally inhibits in-vitro growth of *P.falciparum*, with or without contribution from monocytes, making glurp eligible for parasitemia control.[25]

CONCLUSION AND SUGGESTION

The genetic population of *P.falciparum* in the Merauke Hospital containing a higher percentage of multigenotypes compared with monogenotypes indicated the high transmission of malaria in the study area. As long as the population mobility is still high, the genetic transformation will be high and so will the transmission of malaria.

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