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The Genotyping of Glucose 6 Phosphate Dehydrogenase deficiency (G6PD-d) in Malaria Endemic South Central Timor, East Nusa Tenggara, Eastern Indonesia

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Abstract

Approximately 18 million people live in malaria-endemic areas with 218,450 reported confirmed cases and 161 reported deaths in Indonesia. Currently, primaquine (PQ), the 8-aminoquinolines, is still the only drug for radical cure and preventing relapse of malaria. However, the individuals with G6PD deficiency (G6PD-d) have risk of hemolysis. Currently, few data of the prevalence of G6PD-d and genotyping are available. This study will provide the prevalence of G6PD-d and the genotyping in malaria cases in South Central Timor (TTS) district. G6PD status was analyzed with quantitative (Randox G6PD test, UK) follow with PCR-RFLP and sequencing to identify the variant of G6PD-d genotyping. Malaria was confirmed by n-PCR (Promega, Madison, USA). A total 64 of 181 individuals with G6PD-d from South Central Timor (TTS) district were analyzed. About 25 of 64 cases of G6PD-d were tested positive for malaria with *P. vivax* as the dominant species 56% (14/25) and most of the cases were female 73.3% (11/15). Among the 64 G6PD-d the genotyping *Vanua Lava* (10,883 T>C) WHO classifies G6PD-d genetic variants class II with severe deficiency <10% the enzyme activity were dominant. The variant of *Vanua Lava* is dominant and the high G6PD-d indicated that screening for G6PD deficiency is necessary.

Keywords: G6PD-d, endemic malaria, Eastern Indonesia

1. Introduction

Glucose-6-phosphate dehydrogenase deficiency (G6PD-d) is an X-linked genetic disorder that impacts insufficient enzyme activity. Moreover, 140 allelic variants of G6PD-d mutations are known and has been published [1]. The World Health Organization (WHO) divided G6PD-d into five classes according to the level of enzyme activity in the red blood cells (RBC), and the clinical manifestations [2].

The importance from the variant of genotyping G6PD-d particularly for screening the risk of haemolytic anaemia is induced by the antimalarial primaquine (PQ) although the relationship between G6PD-d with protective to malaria infection is still unknown [3].

The gene of G6PD is an X-linked recessive hereditary disease characterized by abnormally low levels of glucose-6-phosphate dehydrogenase, a metabolic enzyme involved in the pentose phosphate pathway, especially important in red blood cells (RBC) metabolism [4]. Haemolytic anaemia in G6PD-d can be triggered by a range of oxidative agents, such as infections and certain foods and drugs, including antimalarial primaquine [5, 6].

During 2016–2017, the WHO reported that more than 16.7 million population in Indonesia were still living in high malaria transmission areas and the epidemiological distribution of malaria confirmed cases were 218,450 with 161 deaths cases reported. The identification was by *Plasmodium* species: *P. falciparum* 62% and *P. vivax* 37%. Up to date in Indonesia, the only one available antimalarial treatment for radical cure is PQ with 0.25 mg/kg, unfortunately with unknown variant genotyping of G6PD-d [5]. However, a limited study data on the prevalence and variant genotyping of G6PD-d are available in Indonesia, especially in malaria endemic areas. Tantular et al. 2010 studied the genotyping of G6PD-d among students of 7–12 years old in Maumere district, Flores Island, and found that the prevalence of G6PD-d was 4.4% (16/363) and also found there were five different variant genotyping of G6PD gene mutation although in a small population: 31% (5/16) *Coimbra* 592 C>T, and 18.7% (3/16) *Kaiping* 1388 G>A; other case variants are *Vanua Lava* 383 T>C, *Viangchan* 871 G>A, and *Chatam* 1003 G>A, each one cases [4]. Satyagraha et al. 2015 studied that G6PD-d in Sumba District, Eastern Indonesia was 5.1% (104/2033) with the mean average; the activity of G6PD enzyme was <4.6 U/gHb, normal 10 U/gHb; however, in contradiction with others' studies, the dominant genotypes were 98.5% (69/70) variant *Vanua Lava* 383 T>C [3]. Because G6PD-d caused chronic non-spherocytic haemolytic anaemia if PQ is consumed, and for this reason, the variant genotyping must be identifiable in the endemic malaria areas; moreover, most of the G6PD-d are asymptomatic [7].

This study determines the prevalence of G6PD-d and the genotypes of G6PD mutation variants of malaria cases in malaria endemic, Eastern Indonesia, in part, upon previous reviews of variant G6PD-d genotyping by authors, and these may be consulted for more detailed information.

2. Methods

This study was undertaken at five sites (districts) with different levels of malaria endemic in East Nusa Tenggara Province (NTT), Eastern Indonesia. Ethic Approval for this study was approved by ethical committee of the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia, with reference no: KE/FK/85/EC. Data were collected from August 2014 to September 2014, and the whole blood samples were taken from the previous study of Hutagalung et al. [8, 9]. The criteria of inclusion were positive malaria from thick and thin blood smears by Giemsa 3–5% and nested PCR [10], more than 14 years of age, and signed a written informed consent. The study areas were selected by Annual Parasites Incidence (API) based on Health office data 2013/2014 [4]. All participants were interviewed face to face with standard questionnaire, and before enrolment study, all the participants had a physical examination by local health practitioners.

A total of 64 samples diagnosed with G6PD-d were defined by a quantitative G6PD-d test calculated and read at 340 nm/min with a spectrophotometer

according to the manufacturers' protocols from the Randox G6PD test, UK, cat no: PD-410; normal G6PD is <6 U/gHb. The calculation of G6PD activity is as follows: G6PD mU/grHb = mU eritrosit per mL × 100/Hb g/dL. All the assessments of G6PD-d were observed with the G6PD normal control [11]. The assessment of the activity G6PD enzyme is as follows:

The DNA for G6PD genotyping was extracted from the frozen (−20°C) EDTA blood samples and the PCR using the Promega, USA (cat no. A-1120) [12], DNA Mini Kit manufactories protocols. G6PD gene PCR amplification and variant detection using five sets specific primers (25 mM) only for G6PD-d variant from exon 5, 6, 9, 11 and 12 because the reason most common variants in Asia Nguyen et al. [13]. The total volume of PCR G6PD gene was performed in 30 µL total reaction (15 µL green master mix PCR, 1 µL each forward and reverse primer, 2 µL DNA template, and 11 µL nuclease free water/ddH₂O). PCR temperature condition was followed by time melting (T_m) calculation. Positive and negative controls from known samples. The electrophoresis using 100 V and 55–60 minutes was run on agarose 1.5–2% from the Bioron-604001, Germany, containing 5 µL ethidium bromide, Promega, Madison, USA, cat. no. H-5041. The DNA ladder is of (2–3 µL) 100 bp, from the Vivantis, Selangor, Malaysia, cat. no. NL-1407. Finally to identified of genotyping of variant G6PD-d 25 µL PCR product for sequencing to Macrogen Laboratories and all the sequence of each samples was compared to the GenBank accession reference no. X-554481 [6]. The study flow was explained in **Figure 1** and **Table 1**.

While the identification of the *Plasmodium* was using DNA isolation from the whole blood samples, which were collected from each participant, transferred in EDTA anticoagulant tube BD Vacutainer 5 mL, the identification of *Plasmodium* using double assignment microscopic test by Giemsa 3–5% followed nested PCR with five sets of primers (20 mM) with time melting PCR condition followed from Snounow et al. [10, 12].

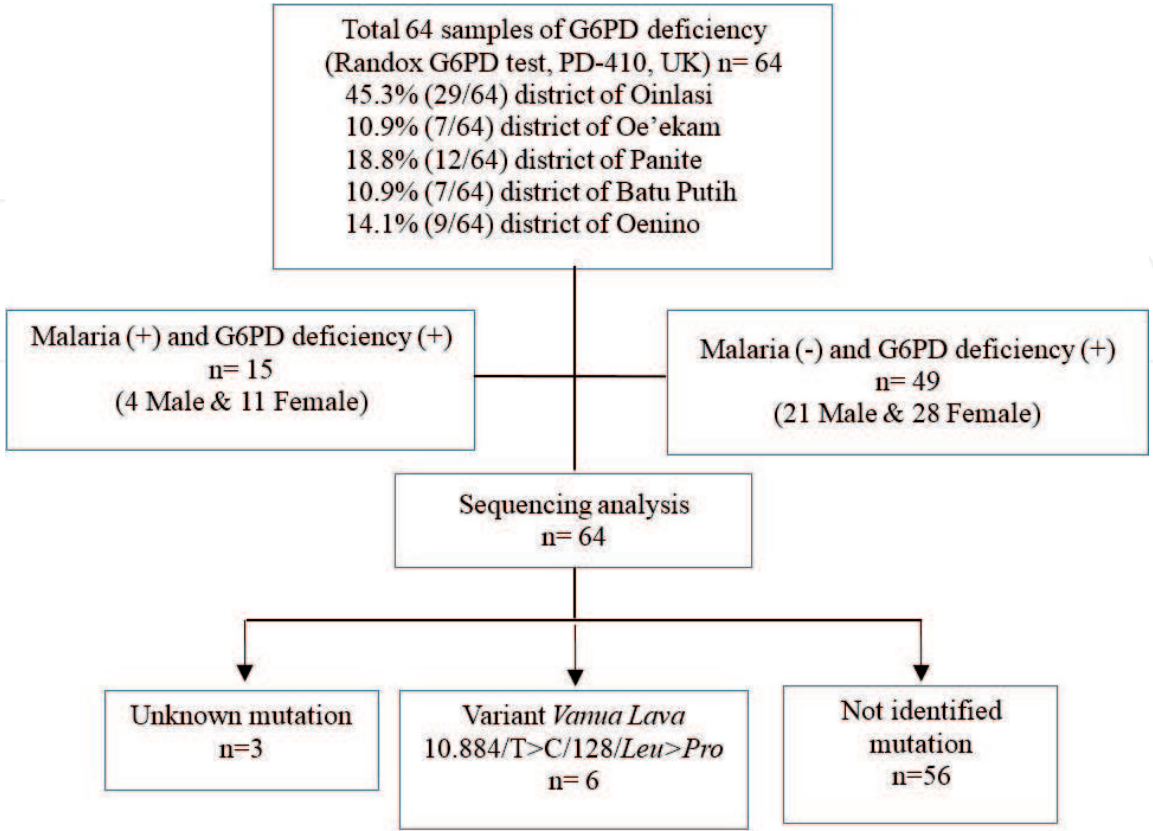


Figure 1.
The study flow.

Results of G6PD (U/gHb)	(+) Malaria/%	(−) Malaria/%	Total/%
Severe deficient 0–3	6/24	19/76	25/39
Moderate deficient 4–6	9/8.8	30/11	39/61
Total	15/23.4	49/76.6	64/100

Table 1.
Results of G6PD quantitative test ($n = 64$), G6PD deficiency test from Randox G6PD test, UK, cat no: PD-410.

3. Results

A total of 64 samples were detected; G6PD-d samples from five district study areas in South Central Timor District, East Nusa Tenggara Province, the Eastern Indonesia were included (**Figure 2** and **Table 2**), in which the mean of age was 42 years old (16–80 years old). During the study, 8.3% (15/181) samples with G6PD-d were infected with malaria. The result of this study showed that the mean average of the activity of G6PD enzyme of the samples was <10.4 U/gHb. The haemoglobin were no different between G6PD-d and normal 13.4 gr/dL (11–17 gr/dL). Almost all the samples were original from Timor ethnicity 99.8% (63/64). The prevalence of malaria in G6PD-d was 8.3%, in which *P. vivax* was the dominant species. However, this study results that the mix infection of malaria in G6PD-d were the *P. falcifarum* and the *P. vivax* infection which was 16.6% (3/15).

This map presents the distribution of the samples were G6PD-d in eastern Indonesia follows the malaria cases. The biggest cases were found in Oinlasi district 45.3% (29/64). However, all the sub-district also present the G6PD-d cases.

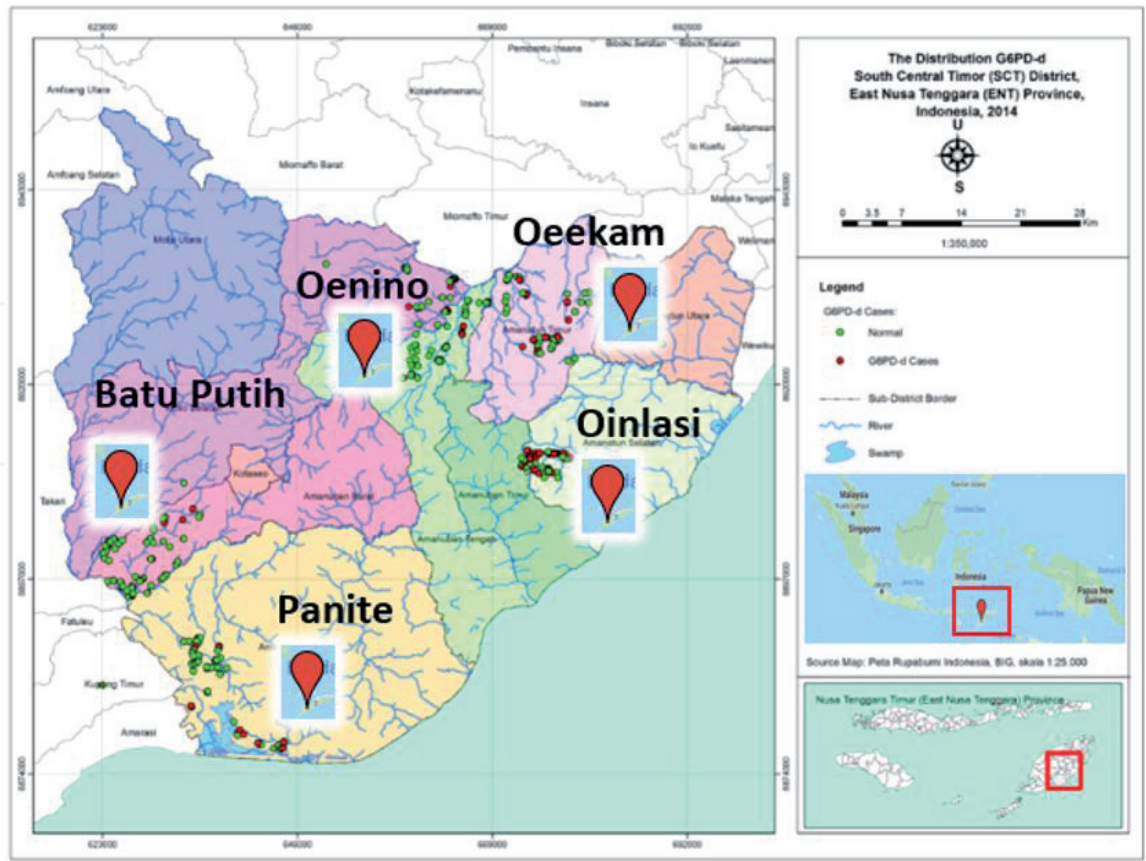


Figure 2.
Map of the five study areas and the distribution of the samples in South Central Timor District, East Nusa Tenggara, Province (Prov. NTT), Eastern Indonesia.

Mutation	Variant	Location	Amino acid	Enzyme activity
10.884 T>C	<i>Vanua lava</i>	Exon-5	128 Leu > Pro	<10% normal
—	—	Exon-6		
13.153 T>C	Unknown	Exon-9	372 Ser > Pro	<10% normal
—	—	Exon-11		
—	—	Exon-12		

Table 2.
The G6PD genotypes in South Central Timor District, East Nusa Tenggara Province, Eastern Indonesia.
GenBank accession reference no. X-554481 [6].

Across the samples of G6PD-d, this study found that the significant 39% (25/64) was severe G6PD-d with the result of quantitative test <3 U/gHb. However, in line with this study, were also it was found that 23.4% (15/64) G6PD-d was due to malaria infection.

Totally, we analysed 64 samples with positive G6PD-d from five districts in Eastern Indonesia randomly. Among 15 samples with G6PD-d positive malaria dominantly were female 73.3% (11/15); these results in line with 76.5% (49/64) of the G6PD-d were also female 57.1% (28/49).

From the sequence analysis result most of the sequence we can not identified the mutation of the samples only 9.4% (6/64) identified with variant G6PD-d *Vanua Lava* (10.884 T>C) amino acid substitution and we also founded 4.6% (3/64) with unknown mutation. G6PD genotypes dominant were *Vanua Lava* (10.884 T>C) in exon 5 with amino acid substitution and unknown mutation 13.153 T>C in exon 9, respectively.

4. Discussion

This study findings 9.4% G6PD-d prevalence with variant *Vanua Lava* 10.884 T>C were dominant (WHO classifies in to class II with severe deficiency <10% the enzyme activity). This study result relevant with the previous study of G6PD-d prevalence in Asia among malaria patients from public health centre in Myanmar was 19.8% (50/252), and at the endemic malaria population areas in Sri Lanka 10.9% (225/2059), showing the prevalence; however, very different to the result of variant genotyping for G6PD-d the most dominant are variant *Mahidol* 487 G>A and *Kaiping* 1388 G>A, the varied in of G6PD-d we notion caused of the different of region and the ethnic status [14, 15]. Unfortunately, this study resulted with the prevalence more higher than the previous study in the same island and showed that the prevalence of G6PD-d was 5.9% (104/2033); however, this study still significant consisted with the variant genotyping were *Vanua Lava* 10.884 T>C was dominant, caused the same of region and ethnicity [3].

This result imposes a greater complexity to consider such antimalarial PQ therapy as more complicated. From the postulated evolution of G6PD-d, it is possible that the frequency of malaria is lower in patient with G6PD-d, though a protective effect against [14]. This consisted of the G6PD-d lower prevalence 7.8% (15/191) vs. non-deficiency 25.6% (49/191). However, from the previous study, in Indonesia, it was showed that there was significance between G6PD-d with malaria infection $p \leq 0.001$ [3, 16]. We would conclude from the theory and previous study that the individual with G6PD-d would perhaps more prove vulnerable and very sensitive to antimalarial PQ therapy in terms of risk of anaemia haemolytic and

the variant genotyping [15, 17]. Authors suggest that G6PD-d induced haemolytic anaemia within 14 days after drug administration of PQ; to prevent this side effect, screening of G6PD-d is necessary prior to drug administration of PQ in malaria patients. Indeed, screening for G6PD-d prior to PQ therapy would likely must to be required to protect patients diagnosed especially with *P. vivax* infection [12, 17]. High G6PD-d were found in endemic malaria areas from public health perspective it remains screening of G6PD-d testing must have to be taken, because in Eastern Indonesia until currently the combination of the PQ with ACT as the only remains for the first line antimalarial drug for radical cure hypnozoites and control for malaria transmission [18, 10, 19, 20]. Indeed, the risk associated with its use must be minimized during reach elimination phases [11, 9]. Patient with malaria should be tested for the enzyme activities and adequately be informed before administration of PQ after knowing the G6PD-d status of the patient, which is a pre-requisite for prescribing PQ with lower doses for individual. The WHO suggests using an intermittent PQ regimen of 0.75 mg base/kg once a week for 8 weeks to help malaria pre-elimination program smoothly [3, 21].

From this study, the prevalence of malaria based on the nested PCR result 32.6% was found with the *P. vivax* and *P. falciparum* dominants. Malaria is a major public health burden in Eastern Indonesia. High asymptomatic infection revealed in this study implied with the fact that asymptomatic malaria is common in high malarious areas and highlighted that low parasitemia or asymptomatic cases should be identified during implementation of the malaria elimination program [22, 23, 24]. This study suggests the active and passive case findings coupled with periodic mass blood surveys (MBS), case management with effective drugs, vector control and good surveillance more needed [25, 10]. This study also agreed about the prevalence of malaria by n-PCR resulted from the previous study from around in malaria endemic areas in Indonesia, Papua from 2004 to 2013 the study showing the mean average of the prevalence above 5–29.7% and higher resulted in Papua, Indonesia 53.3% (86.799/162.966) and more higher in children vs. adult people [23, 19]. This study is also relevant with the study measurement of the prevalence of malaria in other studies in Eastern Indonesia; but, it was still found that the prevalence was 46.4% (86.797/186.869). This study suggested that malaria infection associated with haematological impact diseases, such anaemia and morbidity also greater mortality caused malaria, therefore the public health very importance to plan how to control strategies in areas where the malaria infection was high [26, 27].

Mapping results almost all for the population at risk show that G6PD-d cases follow with the malaria distribution cases. The malaria risk displaying whole area study site, here additionally advantage this maps provide the base for design of the surveillance strategy and will be fully implemented by targeted inland hotspot for prevent outbreak occurred and endemicity maps were used to estimate real incidence malaria and G6PD-d in areas for pre-elimination began [28, 14]. In summary, our survey of G6PD-d in Eastern Indonesia the variant of *Vanua Lava* (10.884 T>C) was relative common among Asia and Eastern Indonesia. The significant risk for increase of the haemolytic cases after the PQ treatment has potential to induce oxidative stress. G6PD-d assessment should be done before antimalarial drug administration.

5. Conclusion and recommendation

The Prevalence of G6PD-d in South Central Timor District was 11.5% with variant G6PD-d *Vanua Lava* (WHO class II) dominant, means required screen before giving the administration of PQ and further research is needed to identified samples with unknown mutations.

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