

The first evaluation of glucose-6-phosphate dehydrogenase deficiency (G6PD) gene mutation in malaria endemic region at South Central Timor (SCT) district, Eastern Indonesia 2014–2015

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Abstract

Primaquine (PQ) is a key drug in the malaria pre-elimination stage. However, PQ can trigger acute hemolysis for people with G6PD deficiency (G6PDd). In 2013, 15–25 million Indonesian people were infected with malaria, with 30,000–38,000 deaths each year mostly in eastern Indonesia with API= 15.6 %. Recently, the Ministry of Health of the Republic of Indonesia announced a plan to reach the pre-elimination stage based on WHO guidelines. This study assesses whether eastern Indonesia should proceed with the activities of malaria pre-elimination. A total 555 healthy people in five subdistricts in eastern Indonesia were selected by systematic random sampling. All data were collected using a standard questionnaire, physical examination, and laboratory tests. PCR and DNA sequencing protocols followed respective manufacture's instructions. Statistical analysis by bivariate with $\alpha= 0.05$ and 95% CI were performed using the SPSS software package. Based on the nested PCR, the result showed a malaria prevalence of 32.6% with being the dominant species (52.5%). Malaria cases were found in all study sites and not using a bed net was the most significant risk factors with Exp B= 1.54 with 95% CI= 0.99–2.38. G6PDd prevalence was 16.6%, the highest G6PDd ever found in Indonesia with variant molecular dominant 10.883 T>C and one sample with a heterozygous female. Malaria pre-elimination in eastern Indonesia should be delayed. High risk patients should be tested for enzyme G6PD activities before antimalarial administration.

Keywords: G6PDd, *Vanua Lava*, Pre-elimination malaria, Eastern Indonesia

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Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is an X-linked essential enzyme that protects cells from oxidative stress, particularly in red blood cells (RBC). G6PD deficiency (G6PDd), the most common known enzymopathy, is a hereditary genetic defect and it is one of the most prevalent polymorphisms in humans, especially in males (Kyoung *et al.*, 2014). G6PD is an enzyme that plays an important role in protecting cells from oxidative damage by producing nicotinamide adenine dinucleotide phosphate (NADPH) and reducing glutathione (GSH) in the erythrocyte. The G6PD gene is highly polymorphic with almost 400 reported variants, and is more commonly found in males than in females. This has led to the speculation that malaria has been the selection pressure that has favored the maintenance of this potentially deleterious trait. There is some evidence that hemizygous and heterozygous G6PDd may be protected against severe malaria. Most of individuals with G6PDd are normally asymptomatic, however, erythrocyte exposure to oxidative stress causes haemoglobin (Hb) denaturation, and ultimately results in haemolysis. Haemolytic anemia in G6PDd can be triggered by a range of oxidative agents, such as infections and certain foods and drugs, including antimalarial 8-aminoquinolines like primaquine (Luzatto *et al.*, 2001; Beutler and Vullamy, 2002; Clark *et al.*, 2009).

Primaquine is the 8-aminoquinolines, a class of drugs discovered almost 80 years ago. Primaquine is administered to patients infected with (hypnozoites) to prevent relapse as well as with to reduce gametocyte carriage to block transmission (Beutler *et al.*, 1995). People with G6PD deficiency more susceptible to hemolysis when taking primaquine. Recently, the Ministry of Health (MoH) of the Republic of Indonesia announced a plan to reach the pre-elimination stage by 2015–2020 and to be free of malaria transmission by 2030, and that it will use the World Health Organization (WHO) guidelines for pre-

elimination activities. WHO criteria for pre-elimination are a reduction to zero incidence where the annual parasite incidence (API) = <1/1,000 population, no local transmission, and with no risk factors for three consecutive years (Elyazar *et al.*, 2011; Kemenkes RI, 2013; WHO, 2014).

In 2011–2014, there were 4.8 million cases of malaria reported in eastern Indonesia with (62%) and (33%) being the most common species (Elyazar *et al.*, 2011; WHO, 2014). In this region, malaria is the primary cause of mortality with 23,483 deaths. South Central Timor (SCT) District, one of the highest malaria prevalence rates in eastern Indonesia with API = 15.6% for more than 10 years following mother mortality rate (MMR) and infant mortality rate (IMR), twice higher than the national average (Kemenkes RI, 2013; Dinkes Prov. NTT, 2013; Dinkes TTS, 2014).

About 49 of 61 countries had failed to achieve malaria elimination in 2010. A review of resurgence in the Philippines and Thailand and two provinces in Indonesia, (Central Java and Kepulauan Seribu Island) that successfully pre-eliminated because of continued active control programs and passive case finding, and strong initiatives with neighboring countries, such as vector control, available operational drugs, and good surveillance systems (WHO, 2007; Feachem *et al.*, 2010; Maharaj *et al.*, 2012).

Malaria pre-elimination will be possible only with serious attempts to address asymptomatic and chronic infections by both and Currently in Indonesia, the available drugs that can completely clear a human of for radical cure and that can reduce the transmission of malaria parasites are those in primaquine, however, people with G6PDd risk having severe adverse reactions such as hemolysis if exposed to these drugs at certain doses (Syafrudin *et al.*, 2009; Kathy *et al.*, 2010). Since 1989, primaquine has been administered in the Indonesian regions without any G6PDd testing, and to date, no study has been undertaken to ascertain the prevalence of G6PDd in SCT District. This study reports on

G6PDd prevalence and the variant molecular in a malaria endemic region regarding whether eastern Indonesia should proceed malaria pre-elimination activities.

Materials and methods

Ethics statements

This study was approved by the ethics committee of the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta (reference No: KE/FK/85/EC). All participants signed a written consent form and gave their permission for the collection of blood samples.

Samples

A survey was conducted from August 2013 to September 2014. Sample size was calculated using a malaria prevalence of 15.6% in 2013 in South Central Timor District, eastern Indonesia (Lemenshow *et al.*, 1997). Criteria for inclusion were ≥ 14 years of age and hemoglobin (Hb) level ≥ 11 gr/dL by using HemoCue (HemoCue, 2013). Participants were interviewed face to face with a standard questionnaire, while household locations were obtained using the Garmin GPS 76, USA (Garmin International Inc., 2006). All participants underwent a physical examination by local health practitioners. During the interview, thick and thin blood smears (Giemsa 5%) were made for malaria identification (Eijkman Institute for Molecular and Kemenkes RI, 2010).

Determination of G6PD activity

Commercial quantitative G6PD enzyme test kits was used to screen for G6PDd population. G6PD enzyme activities were calculated at 340 nm/min with a spectrophotometer at Biochemistry Laboratories, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta. All protocols to determine G6PD activities were according to manufacturer's instructions from the Randox G6PD test. The reliability of test results was monitored by calibration and including controls provided by the Randox G6PD test. Activity G6PD mU/gHb = mU eritrosit per mL $\times 100$ /Hb g/dL. The normal G6PD activities enzymes = ≥ 6.97 -20.5 U/gHb (Randox Laboratories Limited, 2013).

DNA extraction

Whole blood samples were collected in an EDTA anticoagulant tube, specifically a 5 mL BD Vacutainer 5 mL (BD life science, 2014) and stored at -20°C for molecular assessment. DNA was extracted from 200 μL of whole blood using nucleon genomic DNA extraction kit (Promega Co., 2014).

G6PDd genotype variant detection

G6PD variants were detected using five sets of specific primers (20-25 ng/ μL) from exon 5, 6, 9, 11, and 12 (Table 1). PCR amplification of G6PD gene was performed in 30 μL total reaction, which consisted of 1 μL each primer (20 μM), 2 μL of DNA

Table 1. G6PD primers sequence and annealing temperature ($^{\circ}\text{C}$) for G6PD gene amplification.

Exon	PCR product size (bp)	Annealing temp ($^{\circ}\text{C}$)	Primers sequence
5	343	64,5	5-ACA-CAC-GGA-CTC-AAA-GAG-AG-3 5-TGG-TGG-GAG-CAC-TGC-CTG-3
6	320	65	5-TGG-GAG-GGC-GTC-TGA-ATG-AT-3 5-TGG-TCC-TCA-CGG-AAC-AGG-GAA-G-3
9	333	59	5-TCC-CTG-CAC-CCC-AAC-TCA-AC-3 5-AGT-GCG-TGA-GTG-TCT-CAG-T-3
11	157	65	5-TGG-CAT-CAG-CAA-GAC-ACT-CTC-TC-3 5-CCC-CAT-AGC-CCA-CAG-GTA-TGC-AGG-3
12	205	64	5-TGT-GTG-CCA-CCG-GCC-TCC-CA-3 5-ATG-AGG-TAG-CTC-CAC-CCT-CA-3

Table 2. List of the primers sequences and annealing temperature (°C) for PCR-RFLP amplification.

Variant G6PD	Annealing temp (°C)	Enzyme	Primers sequence	PCR Product size
Viangchan /VC	56	Xba-1	5-TGG-CTT-TCT-CTC-AGG-TCT-AG-3 5-GTC-GTC-CAG-GTA-CCC-TTT-GGG-G-3	126 bp
Chatham /CT	61	Bst-X-1	5-CAA-GGA-GCC-CAT-TCT-CTC-CCT-T-3 5-TTC-TCC-ACA-TAG-AGG-AGG-ACG- GCT-GCC-AAA-GT-3	208 bp
Vanua Lava / VL	65	Eco-N-1	5-CAG-CCT-GGG-GCA-GTG-TCT-GTG- CT-3 5-GCG-GTT-GGC-CTG-TGA-CCC-CTG- GTG-3	366 bp

The enzyme from NEB =

Table 3. The primer sequence used for nested PCR and amplification of the species malaria identification (Snounow 1993)

Primers	Primers sequence	Product size (bp) & annealing (°C)
Ist round PCR	Plasmodium genus detection	
r-PLU-5	5-CTT -GTT-GTT-GCC-TTA-AAC-TTC	1,2 kb/58°
r-PLU-6	5-TTA-AAA-TTG-TTG-CAG-TTA-AAA-CG	
2nd round PCR	Species identification	
r-FAL-1	5-TTA-AAC-TGG-TTT-GGG-AAA-ACC-AAA-TAT-ATT	205 bp/58,5°
r-FAL-2	5-ACA-CAA-TGA-ACT-CAA-TCA-TGA-CTA-CCC-GTC	
r-VIV-1	5-CGC-TTC-TAG-CTT-AAT-CCA-CAT-AAC-TGA-TAC	120 bp/55°
r-VIV-2	5-ACT-TCC-AAG-CCG-AAG-CAA-AGA-AAG-TCC-TTA	
r-OVA-1	5-ATC-TCT-TTT-GCT-ATT-TTT-TAG-TAT-TGG-AGA	800 bp/54°
r-OVA-2	5-GGA-AAA-GGA-CAC-ATT-AAT-TGT-ATC-CTA-GTG	
r-MAL-1	5-ATA-ACA-TAG-TTG-TAC-GTT-AAG-AAT-AAC-CGC	144 bp/55°
r-MAL-2	5-AAA-ATT-CCC-ATG-CAT-AAA-AAA-TTA-TAC-AAA	

template (20-25 ng/ μ L), green master mix PCR 15 μ L and 11 μ L distilled water. The PCR temperatures condition used were 30 cycles of initial denaturation at 95°C for 5 min, annealing for 30 sec (table 1) and a final extension at 72°C for 5 min for 30 cycles (Nguyen *et al.*, 2008). Positive and negative controls were used. The PCR products were resolved by electrophoresis on 1.5% agarose gel, containing 5 μ L ethidium bromide (EtBr) (Promega, USA). DNA ladder was used (Vivantis Tech, Malaysia). G6PD variants were detected by sequencing (ABI Prism 310 genetic sequencer, Macrogen 2014). G6PDd were classified according to WHO G6PDd with five level criteria (Marina *et al.*, 1998).

We also re-confirmed the G6PD mutations that were found by PCR-RFLP

using three variants most common in Asia (Nguyen *et al.*, 2008) (Table 2). PCR-RFLP amplification was performed in 25 μ L total reaction, with all protocols PCR-RFLP following the guidelines of KAPA HiFi, Boston, USA, cat. No. KK-2101 (Kappa Biosystems, 2013; Nicole 2014). The G6PD variants were Vanua Lava 10.884 T>C with enzyme restriction , Viangchan Jammu 13.031 G>A with enzyme restriction Xba-1, and Chatham 13.163 G>A with enzyme restriction BstX-1 (New England BioLabs, 2013).

Plasmodium identification

Identification of species used double assignment, microscopic by Giemsa 5% and nested PCR with ten sets of primers. Nested-1

using primers r-PLU-5 and r-PLU-6 and nested-2 using primers are shown in Table 3.

Total nested PCR reaction 25 μ L, PCR using protocols in accordance with Promega, Madison, USA (Cat. No. M-7122) with PCR condition followed from Snounow (1993). A positive control using known samples of and nuclease-free water is used as a negative control. 1.5% agarose gel from Bioron, Germany (Cat. No. 604001), containing 5 μ l/mL ethidium bromide from Promega, Madison, USA (Cat. No. H-5041) was used for electrophoresis. Bands were visualized by ultra violet illumination with DNA ladder from Vivantis, Selangor, Malaysia, Cat. No. NL-1407.

Statistical analysis

This study was evaluated by univariate, bivariate (chi-square), and multivariate statistical analysis test with $\alpha = 0.05$ and 95% confident interval (CI) using the SPSS 16.0 software package. G6PDd subjects were classified as normal and deficient of G6PD enzymatic activities on the basis of the Randox G6PD test (Ranjit, 1999; Martin, 2000).

Results and Discussion

Demography malaria and G6PD prevalence

A total 555 of 558 individuals who were screened in five subdistricts (Oinlasi, Oe'ekam, Panite, Batu Putih, and Oenino), systematically selected, and completed the questionnaire also provided blood samples for analyses of G6PD status and malaria (Figure 1).

The primary objective of this study was to describe the prevalence of G6PDd in eastern Indonesia. G6PDd prevalence was 16.4% (91/555) and predominately female at 56.5% (52/92). Amongst age groups, 41-51 years old had the highest prevalence with 30.4% (28/92). The current study was higher compared with previous reports from other parts of Indonesia, such as Sumba District (5.1%) and Flores District (7.5%), and Malaysia (3.4%), Vanuatu (6.9%), Korea (0.5%), and

Vietnam (8.7%). Samples were selected from healthy individuals in the general population from particular malaria endemic areas, so it is surprising that G6PDd frequencies are divergent from previous reports. However, there are probably very few regions studied with regards to G6PD testing specifically in Indonesia (Ganczakowski *et al.*, 1995; Ainoon 2002; Nguyen *et al.*, 2009; Matsuoka *et al.*, 2013; Satyagraha *et al.*, 2015).

G6PDd prevalence was higher in three malaria endemic regions, Oinlasi Subdistrict (42.8%), Panite Subdistrict (20.8%) and Oe'ekam subdistrict 16.5% where malaria status level high cases incidence and middle cases incidence. A high G6PDd was found in South Central Timor District, justified malaria transmission were unstable transmission area and high potential occurs malaria outbreak. G6PDd testing needs to be undertaken, because currently only primaquine remains as the drug of choice for radical cure for hypnozoites and controlling malaria transmission, and the risk associated with its use must be minimized during pre-elimination (Nick *et al.*, 2011; Goo *et al.*, 2014). All patients with positive malaria should be tested for G6PD enzyme activities and adequately informed before primaquine is administered. In an individual with G6PDd, primaquine should be prescribed in lower doses. WHO suggested using an intermittent primaquine regimen of 0.75 mg base/kg once a week for eight weeks to help the malaria pre-elimination program (Kemenkes RI, 2010; Gonzalo *et al.*, 2013).

Because the majority of respondents in this study were Timorese, it was also the dominant ethnicity with G6PDd (95.6%). Based on the results of the nested PCR, 22 of the 91 G6PDd cases (24%) were infected with malaria with being the most dominant species.

Another important finding in this study was the prevalence of anemia (Hb = ≤ 11 g/dL) in people with G6PDd (83 cases or 91%) and most respondents with G6PDd (98%) had a history of malaria infection (Table 5). Of the risk factors, five variables were

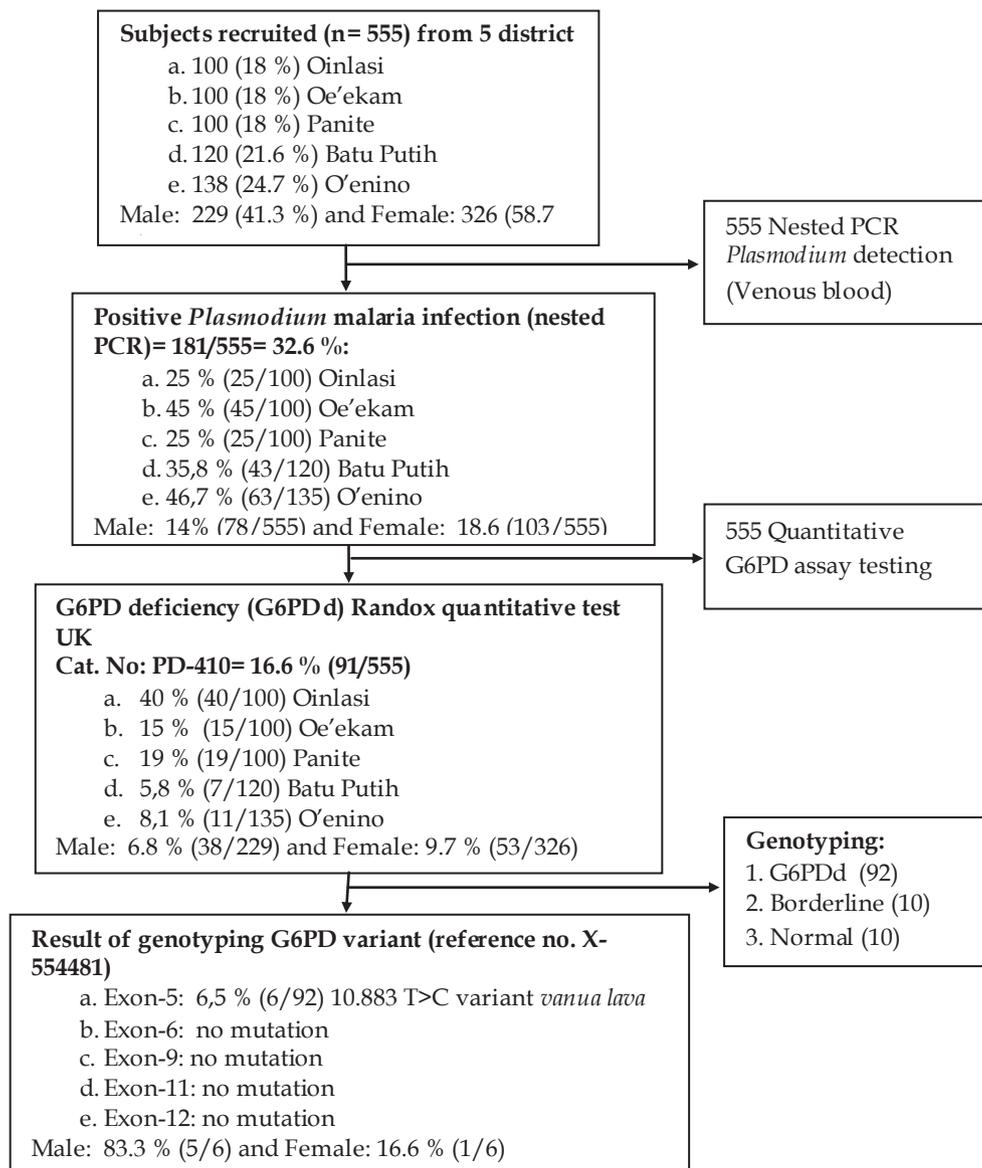


Figure 1. Schematic work flow of this study on malaria prevalence, enzyme activity, and G6PD variants in eastern Indonesia.

significant: decreased Hb level $P = <0.05$ and $OR = 1.26$, abortion history $P = <0.05$ and $OR = 1.04$, antimalarial primaquine consuming $P = <0.05$ and $OR = 1.14$, favism diet $P = <0.05$ and $OR = 1.95$ and malaria infection history $P = <0.05$ and $OR = 1.07$. G6PDd correlated reasonably well with decreased hemoglobin levels, with average G6PDd respondents' mean Hb (11.7 g/dL) lower than the normal group (13.3 g/dL). G6PD activity significant with decreasing hemoglobin levels, in other words, anemia appeared to directly impact

G6PD activity. The explanations for anemia are reticulocytopenia among the anemia and natural decay as red blood cells age. These results are consistent with a study in the National Medical Center and Howard University, Washington DC, USA by Smith *et al.* (2009), which showed that G6PDd had a significant effect on Hb concentration; however, the G6PD activities level was no different in G6PD normal subjects versus those who were G6PD deficient (Jansen *et al.*, 1985; Lorenz *et al.*, 2013; Mehdi *et al.*, 2010).

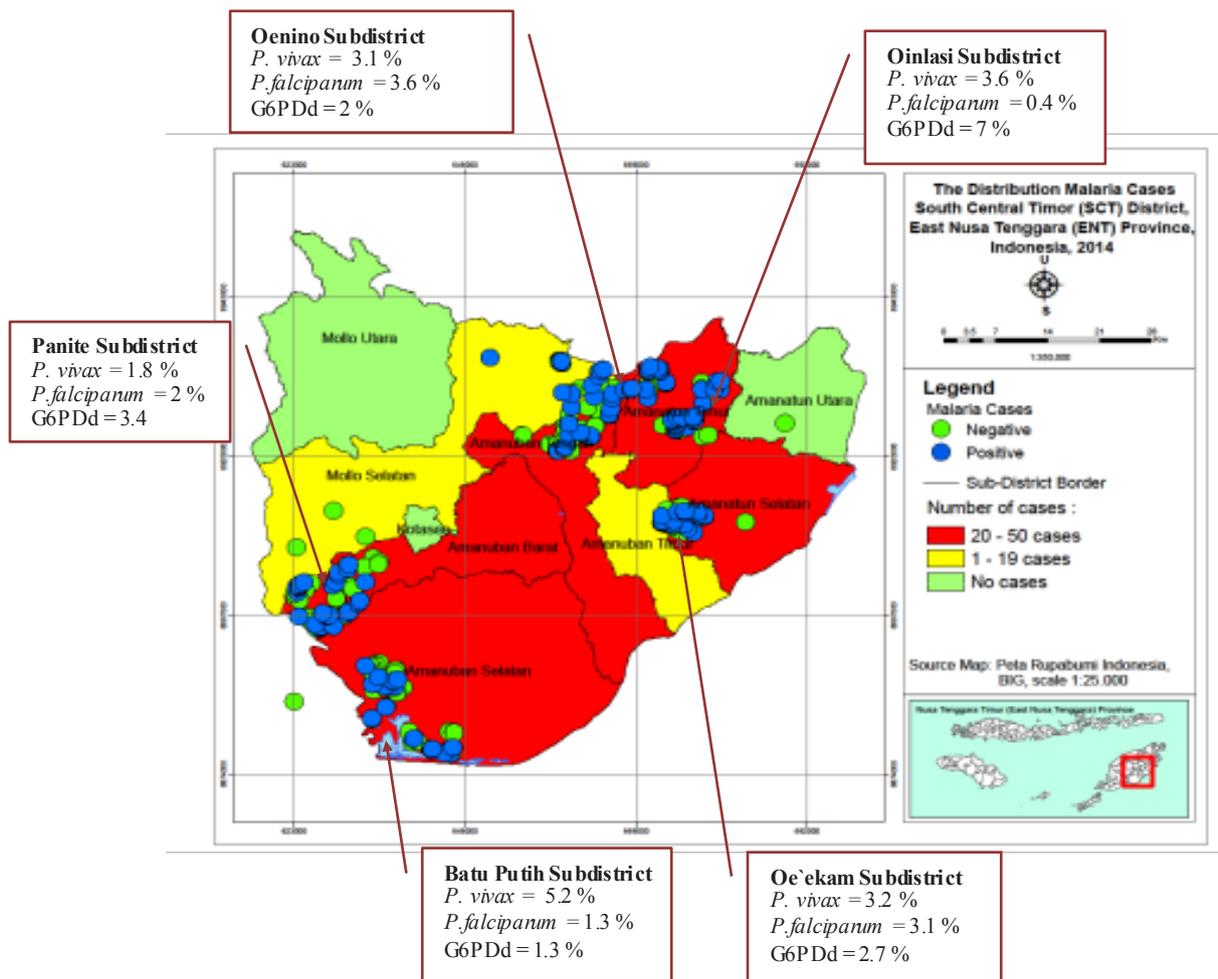


Figure 2. The distribution of the G6PDd and malaria cases in Timor Tengah Selatan district, Nusa Tenggara Timur Province, Indonesia

The prevalence of malaria by nested PCR was higher than that based on microscopic results; 32.6% (181/555) and 1.6% (9/555), respectively, with *P. vivax* as the most dominant. There were more females (57%) than males (43%) infected by malaria and the age group with highest malaria infection was ≥ 51 years (35.9%). Overall, malaria cases were found in all districts and even in districts with a low cases incidence (LCI).

Even the prevalence of malaria was high and no variables significant ($p > 0.05$) to the malaria risk factors, but the OR = >1 , in this study most of eastern Indonesia is living with endemic. However, blood surveys for this parasite are unlikely to detect the dormant liver stage of infection, so the prospects

for pre-elimination would be improved by combining G6PDd distribution, antimalarial therapy practices, and manageability of malaria infection risk (Carlos *et al.*, 2008; Hay *et al.*, 2007; Howes *et al.*, 2012;).

G6PD Gene Genotype

We also screened the nucleotide substitution position which is absent from data based on G6PDd variant (WHO and Minucci *et al.*, 2012). Sequence analysis presents a summary of molecular across five exons of the G6PD gene (5, 6, 9, 11, and 12). Genotype was available for 60.9% (56/92). However, only 8.9% (6/56) identified with the variant (10.884 T>C) with one sample being a heterozygous female (Table 8).

Table 4. Distribution of G6PDd (quantitative test), South Central Timor District, Eastern Indonesia.

Variables	G6PD-d result (quantitative)		Total (%)
	Deficiency (%)	Non deficiency (%)	
Sex:			
Male	40 (7.2)	189 (34.1)	229 (41.3)
Female	52 (9.2)	275 (49.5)	326 (58.7)
Age groups (years):			
< 15	0 (0)	11 (2.0)	11 (2.0)
16-20	2 (0.4)	12 (2.2)	14 (2.5)
21-30	18 (3.2)	63 (11.4)	81 (14.6)
31-40	17 (3.1)	136 (24.5)	153 (27.6)
41-50	28 (5.0)	105 (18.9)	133 (24.0)
> 51	26 (4.7)	137 (24.7)	163 (29.4)
Subdistrict:			
Oinlasi	39 (7.0)	61 (11.0)	100 (18.0)
Oe'ekam	15 (2.7)	85 (15.3)	100 (18.0)
Panite	19 (3.4)	81 (14.6)	100 (18.0)
Batu Putih	7 (1.3)	113 (20.4)	120 (21.6)
Oenino	11 (2.0)	124 (22.3)	135 (24.3)
Ethnicity:			
Belunese	1 (0.2)	0 (0)	1 (0.2)
Sumbanese	1 (0.2)	2 (0.4)	3 (0.5)
Rotenese	1 (0.2)	25 (4.5)	26 (4.7)
Sabunese	1 (0.2)	6 (1.1)	7 (1.3)
Timorese	87 (15.7)	425 (76.6)	512 (92.3)
Others*	0 (0)*	6 (1.0)*	6 (1.0)*
Nested PCR result:			
.	5 (0.9)	52 (9.4)	57 (10.3)
.	11 (2.0)	84 (15.1)	95 (17.1)
and	6 (1.1)	23 (4.1)	29 (5.2)
	0 (0)	0 (0)	0 (0)
Negative	69 (12.4)	305 (55.0)	374 (67.4)
Haemoglobin level:			
Normal (11 gr/dl)	8 (1.4)	197 (35.5)	205 (36.9)
Anaemia (<11 gr/dl)	83 (15.0)	267 (48.1)	350 (63.1)
History of infected malaria:			
Yes	2 (0.4)	37 (6.7)	39 (7.0)
No	89 (16.0)	427 (76.9)	516 (93.0)
Total	91 (16.4)	464 (83.6)	555 (100)

An expected result we found was female heterozygous, 48 years old with lower range of enzymes G6PD activity <3.9 U/grHb, and negative result malaria. This displayed a wide range of G6PD variant in eastern Indonesia. Heterozygosity imposes greater complexity to the consideration of primaquine therapy. From the postulated evolution of G6PDd, it is possible that the frequency of malaria is lower in patients with G6PDd (White *et al.*, 2012). We would conclude that heterozygous G6PDd females

could perhaps prove vulnerable and very sensitive to antimalarial primaquine therapy in terms of risk of hemolytic anemia. Indeed screening for G6PDd prior to primaquine therapy would likely be required to protect patients diagnosed with a infection (Marla *et al.*, 2009).

Malaria pre-elimination program in eastern Indonesia

The malaria pre-elimination activities in eastern Indonesia should be delayed because

Table 5. Bivariate analysis on G6PDd risk factors ($\alpha= 0.05$ with 95% CI).

Variables	G6PDd result		OR/ P-value	95% CI Lower-Upper
	(+) deficiency (%)	Normal (%)		
Malaria endemic				
malaria:				
≤ 5 years	12 (2.2)	60 (10.8)	1.04 (0.53)	0.89-1.12
≥ 6 years	79 (14.2)	404 (72.8)		
Haemoglobin (Hb) level:				
Anaemia (<11 gr/dl)	83 (15)	267 (48.1)	1.26 (0.00)*	1.18-1.34*
Normal (± 11 gr/dl)	8 (1.4)	197 (35.5)		
Abortion history:				
Yes (+)	4 (0.7)	54 (9.7)	1.04 (0.02)*	1.04-1.22*
No (-)	87 (15.7)	410 (73.9)		
History antimalarial:				
Yes (+)	2 (0.4)	37 (6.7)	1.14 (0.03)*	1.05-1.24*
No (-)	89 (16)	427 (76.9)		
Favism diet:				
Yes (+)	19 (3.4)	47 (8.5)	1.95 (0.05)*	1.26-3.02*
No (-)	72 (13.0)	417 (75.1)		
Others Infection history:				
Yes (+)	39 (7.0)	183 (33)	0.97 (0.31)	0.90-1.05
No (-)	52 (9.4)	280 (50.5)		
Malaria infected:				
Positive (+)	22 (4.0)	159 (28.6)	1.07 (0.03)*	1.00-1.15*
Negative (-)	69 (12.4)	305 (55)		

* $p < 0.05$ (significant)/other infections=Thyroid, dengue and chikungunya/
OR=odds ratios/CI 95%=confident interval.

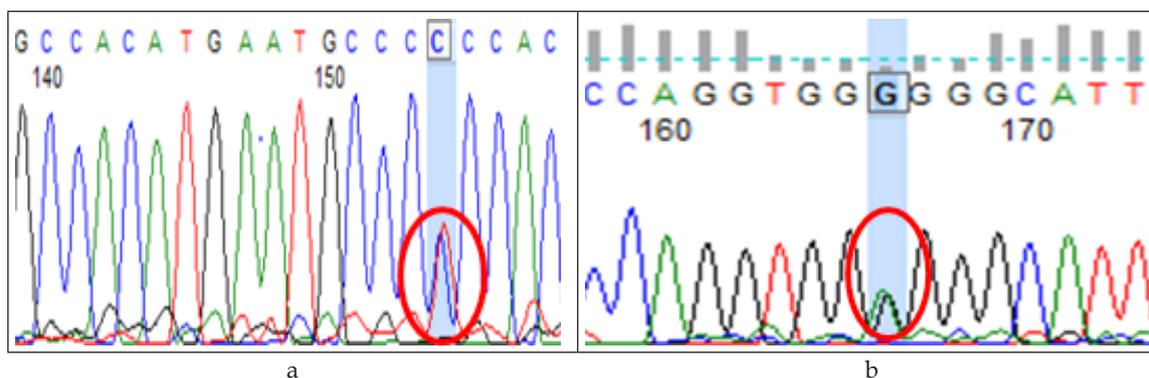


Figure 3. DNA sequencing result of heterozygous female G6PDd, female (SCT-233), 48 years old, negative malaria, (a) forward primer, and (b) reverse primer. Nucleotide substitution 10.884 C>T at codon 128 exon-5.

they do not meet the WHO guidelines of an API of <1/1,000 population (Rajendra *et al.*, 2012). The prevalence of malaria based on this study by nested PCR (32.6%) was still high compared with the microscopic test result (1.6%), with dominating. In order to achieve pre-elimination according to WHO, API must reach <1 case per 1000 people, unfortunately this assessment's results were

not consistent with the national plans for pre-elimination by 2015–2020 (Kemenkes RI, 2010). Malaria is a major public health burden in eastern Indonesia affecting vast regions. Based on a health office report from 2005 to 2010, there were outbreaks in 19 provinces, covering 65 districts with 58,152 cases and 536 reported deaths CFR = >0.9% (Elyazar *et al.*, 2011).

Table 6. The distribution of malaria cases by nested PCR result.

Variables	Malaria infections based on by nested PCR			Total infected (%)	Total (%)
	<i>P. falciparum</i> (%)	<i>P. vivax</i> (%)	<i>P. falciparum</i> and <i>P. vivax</i> (%)		
Sex:					
Male	23 (29.5)	43 (55.1)	12 (15.4)	78 (34.1)	229 (41.3)
Female	34 (33)	51 (49.5)	18 (17.5)	103 (31.6)	326 (58.7)
Age groups (years):					
≤ 15	1 (33)	2 (67)	0 (0.0)	3 (27.3)	11 (2.0)
16-20	3 (75)	0 (0.0)	1 (25)	4 (28.6)	14 (2.5)
21-30	6 (33.3)	7 (33.9)	5 (27.8)	18 (22.2)	81 (14.6)
31-40	15 (31.9)	25 (53.2)	7 (14.9)	47 (30.7)	153 (27.6)
41-50	8 (18.2)	30 (68.2)	6 (13.6)	44 (33.1)	133 (24.0)
≥ 51	24 (36.9)	30 (46.1)	11 (16.9)	65 (39.9)	163 (29.4)
Subdistrict:					
Oinlasi (HCI)	2 (8)	20 (80)	3 (12)	25 (25)	100 (18.0)
Oe'ekam (HCI)	17 (37.8)	18 (40)	10 (22.2)	45 (45)	100 (18.0)
Panite (MCI)	11 (44)	10 (40)	4 (16)	25 (25)	100 (18.0)
Batu Putih (LCI)	7 (16.3)	29 (67.4)	7 (16.3)	43(35.8)	120 (21.6)
Oenino (LCI)	20 (31.7)	17 (26.9)	6 (9.5)	63 (46.7)	135 (24.3)
Ethnic:					
Timorese	54 (32.5)	84 (50.6)	28 (16.8)	166 (32.4)	512 (92.3)
Sabunese	1 (33.3)	2 (66.7)	0 (0.0)	3 (42.8)	7 (1.3)
Rote	2 (18.2)	8 (72.7)	1 (9.1)	11 (42.3)	26 (4.7)
Bugis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0)	1 (0.2)
Sumbanese	0 (0.0)	0 (0.0)	0 (0.0)	0 (0)	3 (0.5)
Others*	0 (0.0)*	0 (0.0)*	1 (100)*	1 (16.7)	6 (1.0)*
Length of stay (years):					
≤ 5	50 (31.4)	82 (51.6)	27 (16.9)	159 (32.9)	483 (87.0)
≥ 6	7 (31.8)	12 (54.5)	3 (13.6)	22 (30.5)	72 (13.0)
Haemoglobin (Hb):					
1. Anemia (≤10 g/dl)	34 (30.4)	58 (51.8)	20 (8.4)	112 (32)	350 (36.9)
2. No anemia (>10 g/dl)	23 (33.3)	36 (52.2)	10 (14.5)	69 (33.6)	205 (36.9)
Antimalarial drug history:					
1. Yes	5 (38.5)	6 (46.2)	2 (15.4)	13 (33.3)	39 (7.0)
2. No	52 (30.9)	88 (52.4)	28 (16.7)	168 (32.6)	516 (93.0)

* Others=Sundanese, Sundanese, Batak and Chinese/* Malariae and ovale negative for all survey participant/HCI= high cases incidence API>5%/MCI= middle cases incidence= API 2-4%/LCI= low cases incidence= API <1%.

To address malaria pre-elimination many interventions have been carried out. Residual spraying with insecticides (initially DDT), rapid diagnostic tests, artemisinin-based combination therapy (ACT), and surveillance have been introduced since 2005 (Noor *et al.*, 2010). In successful cases in Central Java Province, Jembrana (Bali Island), and Sabang District (Aceh Province) intervention focused solely on malaria pre-elimination with seven recommended steps:

(1) producing a map of endemicity and foci of malaria, (2) assessing the feasibility of collaboration between communities and government, (3) developing a strategic plan for malaria control, (4) support from the district health office and legislative council, (5) developing an integrated working plan, (6) implementing the working plan, and (7) monitoring and evaluating the strategy and the progress made (Omumbo *et al.*, 2005; Herdiana *et al.*, 2013).

Table 7. Bivariate analysis on malaria risk factors in eastern Indonesia.

Variables	Nested PCR result (<i>Plasmodium</i> identification)		Odds Ratios (OR)	95% CI Lower-Upper
	Positive (%)	Negative (-)		
Sex:				
Male	78 (14.1)	151 (27.2)	1.07	0.84-1.37
Female	103 (18.6)	223 (40.2)		
Visited others district:				
Yes	76 (13.7)	154 (27.7)	1.02	0.80-1.30
No	105 (18.9)	220 (39.6)		
Using bed nets:				
Yes	146 (26.3)	274 (49.4)	1.52	0.98-2.35
No	35 (6.3)	100 (18)		
Sleeping out of the house at night (field):				
Yes	46 (8.3)	94 (17)	1.01	0.77-1.33
No	134 (24.2)	280 (50.5)		
Screen ventilation used:				
Yes	3 (0.5)	2 (0.4)	1.85	0.89-3.83
No	177 (32.0)	371 (67.1)		
Living near cattle shed:				
Yes	124 (22.3)	251 (45.2)	1.04	0.80-1.35
No	57(10.3)	123 (22.2)		
Mosquitos breeding places near home:				
Yes	144 (25.9)	306 (55.1)	1.05	0.90-1.22
No	37 (6.7)	68 (12.3)		

* Significant variable (p=<0.05)

Table 8. The sequencing alignment result on G6PD gene mutation (accession reference no. X-554481).

Exon	No. sample mutation (%)	Nucleotide (nt) position substitution	Codon/ Amino acid substitution	Class G6PDd WHO	Variant G6PDd
5	6 (6.4)	10.884 T>C	128 Leu>Pro	II	
6	0 (0)	-	-	-	No mutation
9	0 (0)	-	-	-	No mutation
11	0 (0)	-	-	-	No mutation
12	0 (0)	-	-	-	No mutation

*C=Cytosin/T=Timin/Leu=Leusina/Pro=Prolina

This study suggests that more active and passive case finding coupled with periodic Mass Blood Surveys (MBS), case management with effective drugs, vector control, and good surveillance is needed. The conception is very different and rather more complex in the remaining eastern Indonesia and the absolute feasibility of this goal would need to be further assessed with additional work. The situation is entirely different in other province in Indonesia. Reducing high risk because a lot of remote areas, limited

development of healthcare facilities, systems delivering prompt diagnosis, effective treatment including services are the challenge we should manage to achieve malaria pre-elimination in eastern Indonesia (David *et al.*, 2013).

For the population at risk estimates will allow malaria control managers for vector control interventions, help forecast the number of Long-Lasting Insecticide Treated Nets (LLINs) that need to be procured and distributed. The cost estimates of scaling

up LLIN coverage can also be calculated; however, the distribution of the malaria transmitted and the distribution G6PDd need evaluation before any intervention is scaled up (Carlos *et al.*, 2008). The mapping analysis showed that transmission is not homogeneous. Malaria risk is displayed in a whole area of study site. This map provide the base for designing a surveillance strategy and will be fully implemented by targeted inland hotspot for prevent an outbreak. The endemicity maps were used to estimate real incidence malaria and G6PD in areas for pre-elimination. The new methods with serologic assignment is needed to support the pre-elimination program (Howes *et al.*, 2012; Supargiyono *et al.*, 2013).

There are limitations in this study. When selected participants are from a healthy population we cannot describe the real signs and symptoms due to the effect of G6PDd and malaria. Therefore, this study requires further exploration in prospective studies.

Conclusion and recommendation

In summary, our survey of G6PD in eastern Indonesia variant (10.884 T>C) was relatively common within Asia and eastern Indonesia. Primaquine treatment has the potential to induce oxidative stress, and has a significant risk of causing decreased Hb or adverse hemolysis. Therefore, a G6PD assessment should be conducted before antimalarial drug administration. The malaria pre-elimination program in eastern Indonesia should be delayed. Instead, routine treatment to stop silent transmission, outbreak control, and further tests on G6PDd is required before the administration of primaquinine, and more sensitive laboratory support is needed before the pre-elimination program is carried out.

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