

The first evaluation of glucose-6-phosphate dehydrogenase deficiency (G6PD) gene mutation in malaria-endemic region at South Central Timor (SCT) district, Eastern Indonesia 2015-2016

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Abstract. Primaquine (PQ) is the only licensed drug effective against *P. vivax* for specific hypnozoites and as a key drug in the malaria elimination stage. However, PQ can cause severe hemolysis in G6PD deficient individuals. Unfortunately, few epidemiological data of these disorders was in Indonesia. This study aimed to assesses the prevalence and genotyping variant of G6PDd among the people on malaria-endemic. Blood samples from 555 unrelated subjects in eastern Indonesia were for G6PDd by quantitative test and PCR-RFLP-DNA sequencing. All protocols followed by Promega, Madison, USA. The prevalence of malaria and anemia was 32.6% (181/555) and 16% (89/555) with *P. vivax* dominant species 52.5% (95/181), respectively. Overall, 16.6% (92/555) subjects were G6PD deficient, including 58.7% (54/92) females and 41.3% (38/92). Among the 92 cases G6PD deficient molecularly studied, the genotype variant *Vanua Lava* (T10883C) were detected dominant and unknown G6PD deficient (T-13.154-C) in 3 cases. It was high G6PD deficient in eastern Indonesia indicate that diagnosis and management of G6PD deficient are necessary. Obligatory anti-malaria doses for G6PD deficient individuals, population screening, are needed on endemic malaria in eastern Indonesia.

1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is an X-linked essential enzyme that protects cells from oxidative stress, in red blood cells (RBC). G6PD deficiency, the common known enzymopathy, is a hereditary genetic defect and it is one of the prevalent polymorphisms in humans, particularly in males^[1]. G6PD is an enzyme that plays an important role in protecting cells from oxidative damage by producing Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and reduced glutathione (GSH)



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in the erythrocyte. Most of the individual with G6PDd is normally asymptomatic and causes hemoglobin (Hb) denaturation, ultimately resulting in hemolysis. Hemolytic anemia in G6PDd can be triggered antimalarial (8-aminoquinolines including primaquine)^[2,3,4]. PQ is of interest because induced hemolysis increases in the presence of an individual with G6PDd, which depending on the G6PD variant^[4].

In 2013-2014, there were 4.8 million cases of malaria reported in eastern Indonesia with *P. falciparum* 62% and *P. vivax* 33% being the most common species^[5,6]. South Central Timor (SCT) district, malaria infection is the highest malaria prevalence with API= 15.6‰ for more than ten years^[7]. Since 1989, PQ has been without any G6PDd testing^[8]. Currently, there is no study has been undertaken to ascertain the prevalence of G6PD in SCT district. This study aimed to assess the prevalence and genotyping variant of G6PD deficiency among the people of malaria-endemic eastern Indonesia.

2. Materials and methods

The ethics committee of the Faculty of Medicine, Universitas Gadjah Mada approved this study with reference No: KE/FK/85/EC. Total 555 of 558 samples from August 2013 to September 2014. The sample size was calculated using a malaria prevalence of 15‰ in 2013 in eastern Indonesia^[9]. The Criteria for inclusion were ≥ 14 years of age and Hb level = ≥ 10 gr/dL^[10]. During the interview, thick and thin blood smears by Giemsa 3% were for malaria identification^[11].

Quantitative G6PD test kit was used to screen for G6PD deficiency according to the Randox G6PD test protocols, UK Cat. No: PD-410 (normal G6PD enzymes = ≥ 6.97 -20.5 U/grHb)^[12]. DNA extraction protocols followed by Promega, Madison, USA, Cat. No. A-1120^[13]. Five sets specific primers (20-25 ng) from exon 5, 6, 9, 11 and 12 were identified^[14]. G6PD variants were detected using 25 μ L the PCR products and sequencing by ABI Prism 310 genetic sequencer Applied Bio System, MacroGen, Seoul, Korea. Each sample was compared to the sequence of G6PD gene in GenBank with accession no. X-554481.^[15]

Species *Plasmodium* identification using 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 (25 μ L total PCR reaction)^[16] and nested-2 by using protocols followed by Promega, Madison, USA, Cat. No. M-7122. Statistical analysis with $\alpha=0.05$ and 95% confident interval was with the SPSS 16.0 software package.

3. Results

A total 555 of 558 individuals were enrolled this study. Table 1 shows the G6PDd prevalence were 16.4% (92/555) persons were female dominant 56.5% (52/92) and by aged groups the most (41 to 51 years old) 30.4% (28/92). Hb level average G6PDd respondents higher (mean Hb=13.3g/dl) than normal groups (mean Hb=11.7gr/dl) with ethnic Timorese as the major G6PDd was found 95.6%. Of the 91 G6PDd cases 22 (24%) were infected malaria (*P. vivax* dominant). The prevalence G6PDd were anaemia detected in 83 cases (91%) and most of G6PDd (98%) has malaria infected history.

Table 1. Distribution G6PDd (quantitative test), 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)

District:			
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)
Oeninno	11 (2.0)	124 (22.3)	135 (24.3)
Ethnic:			
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:			
<i>P. falcifarum</i>	5 (0.9)	52 (9.4)	57 (10.3)
<i>P. vivax</i>	11 (2.0)	84 (15.1)	95 (17.1)
<i>P. vivax</i> & <i>P. falc</i>	6 (1.1)	23 (4.1)	29 (5.2)
<i>P. malariae</i> + <i>P. ovale</i>	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 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)

Bivariate resulted (Table 2) in G6PDd risk factors, five variables significant; decreased Hb level ($P<0.05$ and $OR=1.26$), abortion history ($P<0.05$ and $OR=1.04$), antimalarial (PQ) 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 $OR=1.07$).

Table 2. Bivariate analysis G6PDd risk factors ($\alpha=0.05$ with 95% CI).

Variables	G6PDd result		OR ^c / P-value	95% CI ^d Lower-Upper
	(+) deficiency (%)	Normal (%)		
Stay 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 (<11gr/dl)	83 (15)	267 (48.1)	1.26 (0.00) ^a	1.18-1.34 ^a
Normal (±11gr/dl)	8 (1.4)	197 (35.5)		
Abortion history:				
Yes (+)	4 (0.7)	54 (9.7)	1.04 (0.02) ^a	1.04-1.22 ^a
No (-)	87 (15.7)	410 (73.9)		
History antimalarial:				
Yes (+)	2 (0.4)	37 (6.7)	1.14 (0.03) ^a	1.05-1.24 ^a
No (-)	89 (16)	427 (76.9)		
Favism diet:				
Yes (+)	19 (3.4)	47 (8.5)	1.95 (0.05) ^a	1.26-3.02 ^a
No (-)	72 (13.0)	417 (75.1)		
Others Infection history^b:				
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) ^a	1.00-1.15 ^a
Negative (-)	69 (12.4)	305 (55)		

^ap<0.05 (significant)^bOthers infection=Thyroid, dengue and chikungunya^cOR=odds ratios^dCI 95%=confident interval

G6PD genotype sequence analysis presents a summary of molecular across five exons G6PD gene (5, 6, 9, 11 and 12). However only 8.9% (6/56) identified with *Vanua Lava* variant (10.884 T>C) with one sample heterozygotes female and we also screen 91.1% (49/56) the nucleotide substitution position was absent from data based G6PDd variant.

Table 3. Sequencing G6PDd gene mutation (accession 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 ^b >C ^b	128 Leu ^b >Pro ^b	2	Vanua Lava
	1 (1)	^a 10.928 C ^b >T ^b	143 Pro ^b >Leu ^b	3	^a Unknown
	1 (1)	^a 10.931 C ^b >T ^b	144 Pro ^b	3	^a Unknown
6	0 (0)	-	-	-	-
9	9 (8.6))	^a 13.096 C ^b >del ^b	352 Pro ^b	3	^a Unknown
	17 (16.3)	^a 13.113 C ^b >del ^b	358 Ala ^b	3	^a Unknown
	26 (25)	^a 13.114 C ^b >del ^b	358 Ala ^b	3	^a Unknown
	5 (4.8)	^a 13.115 A ^b >del ^b	359 Thr ^b	3	^a Unknown
	5 (4.8)	^a 13.153 G ^b >T ^b	372 Gly ^b	3	^a Unknown
	14 (13.5)	^a 13.154 T ^b >C ^b	373 Ser ^b >Pro ^b	3	^a Unknown
11	1 (0.9)	^a 13.784 G ^b >A ^b	505 Gly ^b >Ser ^b	3	^a Unknown
12	1 (1.1)	^a 13.921 G ^b >A ^b	511 Thr ^b	3	^a Unknown
		^a 13.933 G ^b >A ^b	515 His ^b	4	^a Unknown
		^a 13.967 T ^b >G ^b	531 Gly ^b	4	^a Unknown
		^a 13.970 G ^b >A ^b	534 Gly ^b >Arg ^b	4	^a Unknown

^aUnknown mutation^bA=Adenine/C=Cytosin/T=Thymine/G=Guanine/Ala=Alanine/Leu=Leucine/Pro=Proline/Gly=Glycine/Ser=serine/Thr=Threonine/His=Histidine**4. Discussion**

The primary objective of this study was to describe the prevalence of G6PDd in eastern Indonesia. The prevalence of G6PDd in the current study was higher compared with previous report from Sumba (5.1%), Flores (7.5%), Malaysia (3.4%), Vanuatu (6.9%), Korea (0.5%), and Vietnam (8.7%)^[17,18,19,20]. The current study selected healthy individuals in the general population although particular malaria endemic areas, so it is surprising that G6PDd frequencies are divergent from previous reported. However, there were probably very few as the regions studied to G6PD testing. As expected, we founded female heterozygous displayed a wide range of G6PD variant in eastern Indonesia. From the postulated evolution of G6PDd, it is possible that the frequency of malaria is lower in patient with G6PDd, though a protective effect against^[21]. Across all G6PDd correlated reasonably well with the decreased of Hb. These result are consistent with study in National Medical Center and Howard

University, Washington DC, USA, showed G6PDd significant effect on Hb concentration, however no different G6PD activities level in G6PD normal subjects versus those who were G6PD deficient^[22].

High G6PDd were found from public health perspective remains G6PDd testing need to be taken, because currently the only PQ remains the drug of choice to radical cure (hypnozoites and control) malaria transmission, the risk associated with its use must be minimized during pre-elimination. Patient should be tested for G6PD deficiency and adequately informed before administration of PQ. WHO suggest using an intermittent PQ regimen of 0.75 mg base/kg once a week for eight weeks^[23].

Malaria cases distribution shown that transmission is not homogeneous, 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 in areas for pre-elimination began^[24]. There are limitations in this study, therefore, this study require exploratory in prospective studies.

5. Conclusion

G6PDd *Vanua Lava* (10.884 T>C) was relative common among eastern Indonesia. Significant risk for decreased Hb or adverse hemolytic after PQ treatment that have potential to induce oxidative stress. G6PD assessment should be done before antimalarial drug administration. Further research is needed to identified 58 samples with unknown G6PD gene mutations.

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