MOLECULAR ANALYSIS FOR THE DETECTION OF GLUCOSE 6- PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY

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ABSTRACT

Glucose-6-Phosphate Dehydrogenase is the first and primary enzyme of the regulatory enzymes in the pentose phosphate pathway - the only method by which red blood cells can generate NADPH. Deficiency of G6PD is the most common enzymopathy that affects over 400 million people worldwide. Glucose-6-Phosphate Dehydrogenase deficiency is found in regions where malaria is endemic or has been endemic and it confers resistance against malaria especially malaria caused by *Plasmodium falciparum*. Mutation upon this X-linked gene - encoding for G6PD - causes enzyme deficiency from asymptomatic to chronic nonspherocytic haemolytic anemia. Over 300 biochemical and genetically distinct variants of G6PD deficiency have been described in the past due to the polymorphism of the gene.

This study intends to assess the molecular analysis for the detection of G6PD deficiency in given areas namely Medan, Bangka Island and Palangkaraya, where the level of malaria is not very high but G6PD deficiency is present. Blood samples were collected from apparently healthy males. Glucose-6-Phosphate Dehydrogenase deficiency was screened biochemically by using Sigma fluorescence spot test. DNA was extracted from buffycoat using soluble saturated phenol (ss-phenol) standard method while exons were PCR- amplified. Restriction endonuclease digestion analysis was used to determine known mutations while Single-Strand Conformation Polymorphism (SSCP) followed by DNA sequencing was used to determine unknown mutations. DNA from deficient males was the subject for this study and DNA from normal male was used as control for all analysis.

From 13 deficient male subjects studied, subjects from Bangka Island and Palankaraya showed G6PD deficiency on molecular level. Glucose-6-Phosphate Dehydrogenase Canton variant was found in Bangka. The mutation produces a G→T substitution at position 13884 resulting in the appearance of *XhoI* site in exon 12 and substitution of amino acid Arg→Leu at position 459. However, G6PD Viangchan was found in Palangkaraya. The mutation produces a G→A substitution at position 13031 predicting Val→Met amino acid change at position 291.

Keywords: *G6PD Deficiency, Polymorphism, restriction endonuclease digestion, SSC*

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INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) is the first and primary enzyme of the regulatory enzymes in the pentose phosphate pathway of carbohydrate metabolism, the only method by which red blood cells can generate reduced nicotinamide adenine dinucleotide phosphate (NADPH). The main physiological role of G6PD is to provide NADPH, a compound necessary for a number of detoxification and biosynthetic reactions (Martini *et al.*, 1986). Reduced nicotinamide adenine dinucleotide phosphate is required for the activation of catalase and maintaining glutathione in its reduced form (GSH), which is the substrate of the glutathione peroxidase. Reduced nicotinamide dinucleotide phosphate also is required for maintaining sulfhydryl groups in the red cell membrane. The pathway therefore protects red blood cells against oxidative damage.

Glucose-6-Phosphate Dehydrogenase monomer consists of 515 amino acid subunits with a molecular weight of 59,256 Daltons (Beutler *et al.*, 1994). The active form of G6PD is a dimer or a tetramer consisting of identical subunits. Both forms are active and are in a pH dependent equilibrium with each other, with about equal proportions present at neutral pH (Luzzatto and Mehta, 1989). The smallest active form of the enzyme is a dimer that is tightly bound to NADP. The entire amino acid sequence of human G6PD derived from the cDNA sequence is now known (Takizawa *et al.*, 1986; Orita *et al.*, 1986). Glucose-6-phosphate dehydrogenase sequence for about 24 species, including plants, yeasts, flies, and bacteria are now known. They are all similar in size except G6PD from *Plasmodium falciparum,* which is nearly twice as big as the others. In all species, 34 residues are fully conserved (Fig. 2.) the most highly conserved peptide being the eight totally conserved amino acids including the Lys-205 (Mason, 1996).

The gene that encodes G6PD maps at the long arm of the X chromosome at the Xq28 locus between the gene for the color vision and the clotting factor VIII. It spans over 20 Kb in length and consists of 13 exons and 12 introns. The first exon contains no coding sequence and all introns are small, less than 1 Kb except for intron 2, which is extraordinary long, extending for 9,857 bp. Among males only two categories are observed i.e. normal (XY) and deficient ($X^{-}Y$), while in females there is a third group with intermediate value ($X^{-}X$): heterozygous (Siregar *et al.*, 1989).

Glucose-6-phosphate dehydrogenase deficiency is the most common and known enzymopathy, and is estimated to affect over four hundred million people worldwide, mainly in tropical and subtropical

regions, where malaria is or has been endemic. The vast majority of individuals with G6PD deficiency are usually asymptomatic and go through life without being aware of their genetic abnormality but they are subject to attacks of hemolytic anemia precipitated by certain drugs (the 8- aminoquinoline antimalarial drug primaquine), or foodstuffs like fava bean (*Vicia faba*) called also favism, or infection caused by bacteria, virus or rickettsia. Glucose-6-Phosphate Dehydrogenase deficiency also causes neonatal jaundice and chronic nonspherocytic hemolytic anemia (Beutler *et al.*, 1994).

The frequency of G6PD deficient alleles has reached polymorphic levels in many parts of the world, where malaria is endemic. It is thought that the high frequencies of deficient alleles confer some selective advantage against malaria infection especially malaria caused by *Plasmodium falciparum*.

To date, more than 400 variants have been characterized according to the suggested World Health Organization criteria, while 122 mutations or combination of mutations associated with 177 variants have been characterized at the molecular level (Vulliamy *et al.*, 1997).

Despite the great size of Indonesian populations that represent about a third of the total population of South-East Asia, very limited information is available in the literature on the distribution of G6PD deficiency variants in different populations of Indonesia (Soemantri *et al.*,1995). Soemantri *et al.* (1995) studied the molecular variants of G6PD deficiency in central Java (Variant Mediterranean). Later, Al-Azhar (1998) described the frequency of G6PD deficiency in three populations of Nusa Tenggara Islands. The author showed that the frequency of G6PD deficiency is 6.1%, 5.4%, and 6.6% in Sumbawa, Sumba and Alor population respectively. Kurniatun (2000) described the molecular variant of G6PD deficiency of these populations. The author showed that a 10884T→C change was found in Alor populations.

This study intends to assess the application of restriction endonuclease digestion and single-strand conformation polymorphism to analyze G6PD deficiency at the molecular level in given geographical areas in Indonesia where G6PD frequency of deficiency is very high and malaria level is low.

MATERIALS AND METHODS

The subjects were apparently healthy males from Medan, Bangka, and Palangkaraya. Ten to 15 ml of blood were drawn from antecubital vein using EDTA-venoject Terumo. All blood specimens were kept cool in a domestic refrigerator until air freighted in ice to Yogyakarta for further studies.

Glucose-6-phosphate dehydrogenase activity was tested by using Sigma fluorescence spot test kit Cat. No. 202 to differentiate deficient individuals from normal. DNA was extracted using ss-phenol standard method. DNA from deficient individuals were the subject for this study and DNA from normal was used as control.

Table 1. Primer sequences and annealing temperatures for G6PD exons

Exon	Primer	Sequence	Annealing T (°C)
3,4	→η	CAGCCACTTCTAACCACACACCT	64
	←P4	CCGAAGTTGGCCATGCTGGG	
5	→A	CTGTCTGTGTGTCTGTCTGTCC	64
	←M	GGCCAGCCTGGCAGGCGGGAAGG	
6,7	→91	CCCCGAAGAGGAATTCAAGGGGGT	63
	←92	GAAGAGTAGCCCTCGAGGGTGACT	
8	→L	GGAGCTAAGGCGAGCTCTGGC	65
	←I	GGCATGCTCCTGGGGACTGTG	
9	→D	CAAGGAGCCCAGTCTCCCTT	64
	←R	TGCCTTGCTGGGCCTCGAAGG	
10	→E	CTGAGAGAGCTGGTGCTGAGG	65
	←S	AGGCCGCCCACCCTCCACACT	
11	→T	GCAGGCAGTGGCATCAGCAAC	60
	←Y2	CCCCATAGCCCACAGGTCTGCAGG	
12	→T	GCAGGCAGTGGCATCAGCAAG	58
	←Cant on	TCAAAATACGCCAGGCCTCG	

Exons 3-4, 5, 6-7, 8, 9, 10, 11, 12 were PCR- amplified using primers designated by Sigma Genosys LTD in United Kingdom (Table 1.) Polymerase chain reaction conditions were optimised in an Eppendorf Mastercycle 5330 or BIO-RAD Gene Cycler™ serie No. 10487. A mixture of 5 µl of 10 X PCR buffer, 4µ $MgCl_2$, 1 µl of each primer forward and reverse, 1 µl of 100 ng/µ of dNTPs, 0.15µl of Taq polymerase (10u/µl), 1 µl of DNA template (200ng/µl), and 36.85 µl of distilled water was put into the programmed thermocycler at 95°C for 1 minute for denaturation (except for first cycle that was 7 minutes), annealing temperature (Table 1.) for 1 minute, and extension at 72°C for 1 minute 15 seconds (except for the last cycle that was 7 minutes). These steps were repeated in 34 cycles. After amplification, PCR products were visualized on 2% agarose gel stained with ethidium bromide.

Exons 6-7, 11, 12 were analysed by enzyme restriction digestion (Table 2.). A mixture of 5 µl of PCR product, 1 µl of the appropriate buffer of the enzyme, o.5 µl of enzyme (10 u/µl), and 3.5 µl of distilled water was incubated at 37°C for 5 hours. After completion of incubation, digestion product was visualized on 4% agarose gel stained with ethidium bromide.

Table 2. Variants and enzymes used for G6PD deficiency analysis

	PCR fragment		Restriction-fragment size(s) (bp)		
G6PD variant	Exon(s)	Size (bp)	Enzyme	Wild Type	Mutant
Mediterranean	6,7	583	*Mbo*II	379,120,60	103,276,120,60
Union	11	160	*Hha*I	65,45,50	95,65
Canton	12	253	*Xho*I	253	232,21

Exons 3-4, 5, 8, and 9 were analysed using Single Strand Conformation Polymorphism (SSCP) method. A mixture of 5 µl of PCR product and 5µl of formamide dye was denatured in boiled water for 10 minutes and snap frozen on ice for 5 minutes before electrophoresis on 8% polyacrylamide nondenaturing gel at 150 volts for 3 hours. After completion of electrophoresis, gel was analysed by silver staining method.

RESULTS AND DISCUSSIONS

Eight subjects from Medan, two subjects from Bangka, and three subjects from Palangkaraya were G6PD deficient. Analysis of

PCR product on 2% agarose gel stained with ethidium bromide and visualized under ultra violet light (Fig. 1.) showed the products of all exons of G6PD gene. They were 352 bp, 295 bp, 583 bp, 164 bp, 252 bp, 318 bp, 160 bp, and 253 bp in size for exons 3-4, 5, 6-7, 8, 9, 10, 11, and 12 respectively.

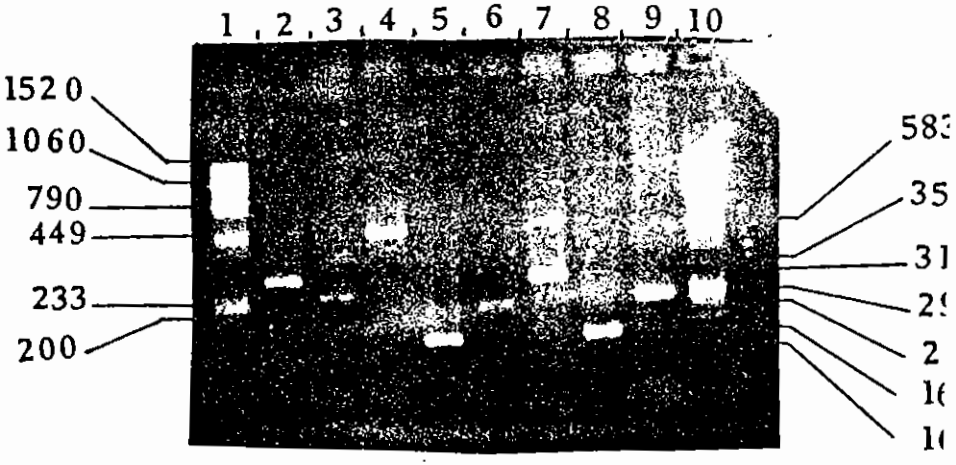

Figure 1. PCR product of the exons of G6PD gene on 2% agarose gel. Lanes: 1 and 10 are size markers (ϕX174 digested with *Hae*III). Lanes 2, 3, 4, 5, 6, 7, 8, 9 are exons 3-4, 5, 6-7, 8, 9, 10, 11, and 12 respectively.

Restriction endonuclease analysis of exon 12 showed a mutation of G6PD Canton on the subject from Bangka. The appearance of 253 bp fragment (Fig. 2.) indicates the existence of an *Xho*I site on the exon. This change is the consequence of the 13884G→T nucleotide substitution on exon 12 which creates the *Xho*I site. The restriction endonuclease digestion of exon 6-7 and 11 did not show any mutation.

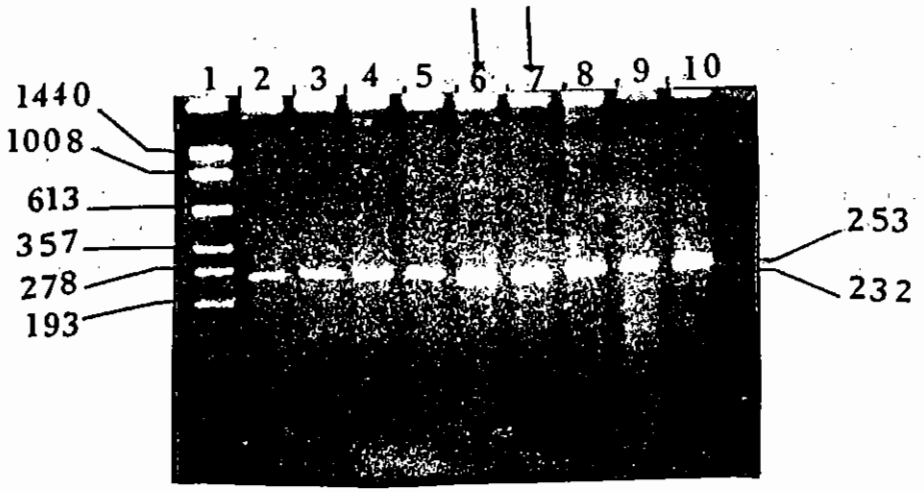

Figure 2. PCR product of exon 12 digested with *Xho*I on 4% agarose gel. Lane 1: pEMBLγ, lanes 2: control cut, lane 3: uncut, lanes 4 and 5: subjects from Medan, lanes 6 and 7: subjects from Bangka, lanes 8-10: subjects from Palangkaraya. Arrows indicate the mutant subjects.

Single Strand Conformation analysis of exon 9 for a subject from Palangkaraya showed an abnormal shift (Fig. 3.). A DNA sequencing analysis for that mutant subject showed a G→A nucleotide substitution at position 13031 predicting a Val→Met amino acid substitution at position 291. This G6PD deficiency variant has once described as G6PD Viangchan in the Laotian people (Poon *et al.*, 1988; Beutler *et al.*, 1991).

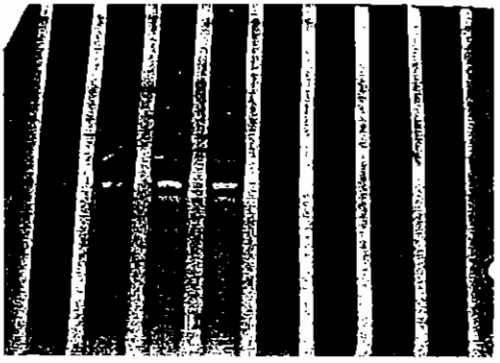

Figure 3. SSCP of exon 9 on 8% polyacrylamide non denaturing gel. Lane 1 and 8: controls, lanes 2-4 are subjects from Medan, lanes 5-7 are subjects from Palangkaraya. Vertical arrow indicates the mutant subject. Horizontal arrow indicates single strand DNA.

The discovery of G6PD Canton and G6PD Viangchan in Indonesia is very interesting and it has been a question that is these mutations are locale mutations for these areas or mutations caused by the gene flow from the mainland Asia during people movement toward the Pacific. Compared with ovalocytosis found in Indonesia populations, which showed the deletion of 27-bp on the gene encoding for band-3 protein common in South East Asia and Oceania populations (Fujiati, 2000), it was thought that these mutations were the consequence of the gene flow from Asia during people movement in the past. Glucose-6-phosphate dehydrogenase variant Canton is found in Bangka thought of the predominance of Mongoloid phenotype in Western Indonesia and G6PD variant Viangchan is found in Palangkaraya thought of the predominance of Proto-Malays phenotype in this region (Bellwood, 1997).

CONCLUSION

From the results of amplification of the exons of G6PD gene using PCR followed with restriction endonuclease digestion analysis and SSCP analysis, mutations on molecular level of G6PD were found in Bangka (G6PD Canton) and in Palangkaraya (G6PD Viangchan) while in Medan, the mutation on molecular level was not found in the deficient subjects. Further studies are needed to characterize the G6PD deficiency variants existing in Indonesia.

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