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Modified fluorescent spot test for determination of G6PD deficiency

Ratana Sindhuphak*
Chaweewan Impun**

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G6PD deficiency is an inherited sex-linked condition in which the activity of red cell G6PD enzyme is markedly diminished. We have modified the fluorescent spot test, one of the appropriate screening method for routine use. This method correlates well with standard quantitative method using enzyme and spectrophotometry. The clinical sensitivities for detection of G6PD deficiency and its intermediate are 92 and 83 per cent, respectively, with 98 per cent clinical specificity. The advantages of this method are high specificity, simplicity, inexpensiveness and high reliability. Moreover, the reagents used in this method are quite stable.

Key words : *G6PD deficiency, Fluorescent spot test.*

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* Institute of Health Research, Chulalongkorn University.

** Department of Pediatrics,Faculty of Medicine, Chulalongkorn University.

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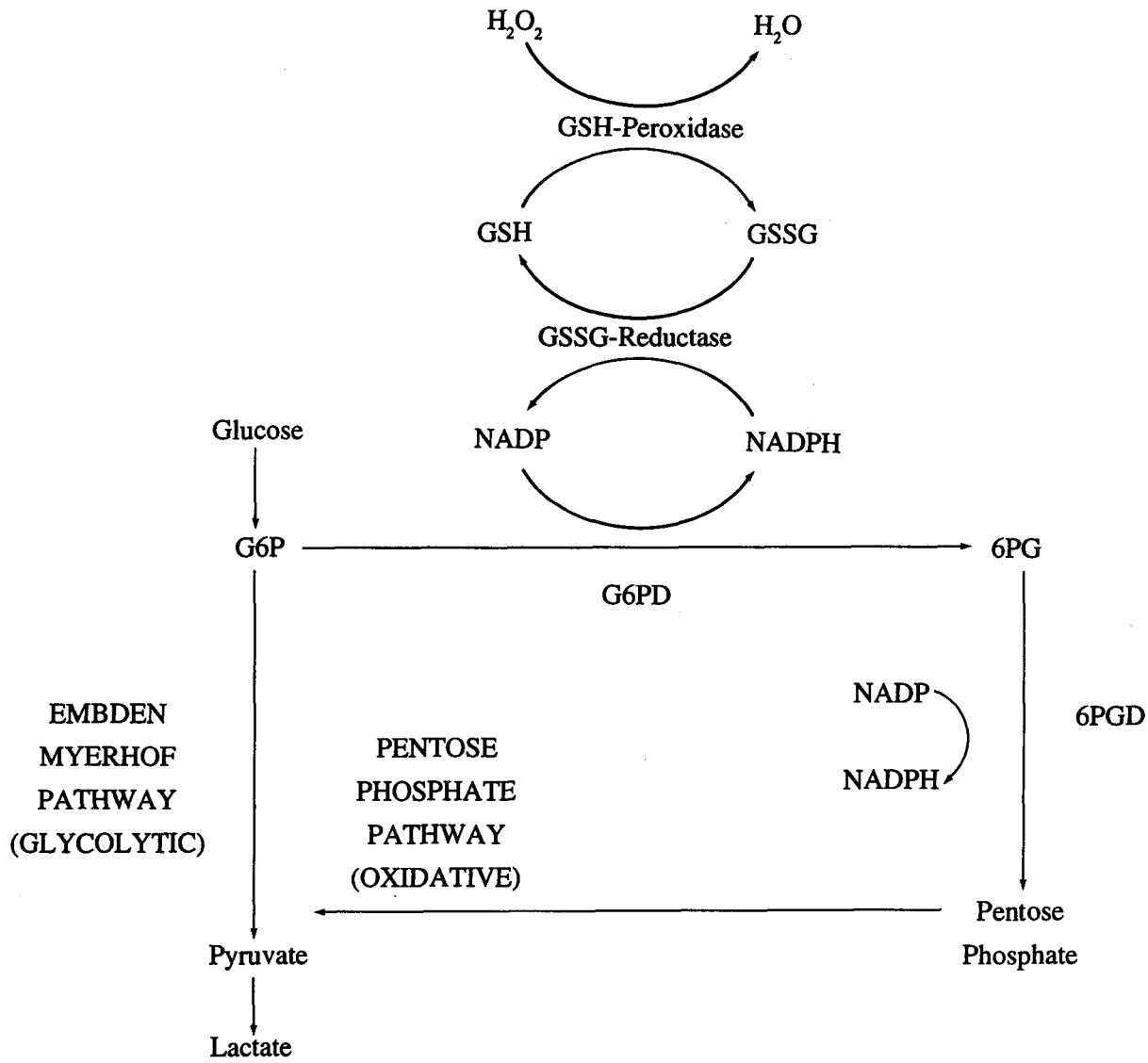
ภาวะพร่องจี 6 พีดี เป็นลักษณะผิดปกติ ทางพันธุกรรม ของ X-chromosome ทำให้ การทำงานของเอ็นไซม์ จี 6 พีดี ลดลง วิธี fluorescent spot test ที่เสนอในรายงานนี้ เป็นวิธีหนึ่ง และเป็นวิธีที่เหมาะสม ที่ใช้ตรวจ ในห้องปฏิบัติการได้ผลดี ใกล้เคียงกับ วิธีมาตรฐาน ซึ่งใช้หลักการของ เอ็นไซม์ และ เครื่องมือสเปคโตรโฟโตมิเตอร์ (quantitative method) มีความไวทางคลินิกในการตรวจภาวะพร่อง จี 6 พีดี และภาวะ intermediate 92 และ 83 เปอร์เซ็นต์ตามลำดับ มีความจำเพาะ 98 เปอร์เซ็นต์ ข้อได้เปรียบของวิธีนี้ คือ มีความจำเพราะสูง วิธีการทดลองง่าย รวดเร็ว ราคาถูก และเชื่อถือได้ นอกจากนี้น้ำยาที่ใช้ตรวจสามารถเก็บไว้ได้นานด้วย

Glucose-6-phosphate dehydrogenase (G6PD) is a ubiquitous enzyme. The metabolic role of G6PD is well outlined (pentose phosphate pathway). It catalyzes the first step of the pathway for producing pentose, a precusor of nucleic acids and nucleotide coenzymes. It also provides the NADPH required for a variety of biosynthetic and detoxification reactions (Figure 1).



G6P = glucose-6-phosphate, **G6PD** = glucose-6-phosphate dehydrogenase
6PG = 6-phosphogluconate, **6PGD** = 6-phosphogluconate dehydrogenase
GSSG = oxidized glutathione, **GSH** = reduced glutathione
NADP = nicotinamide adenine dinucleotide phosphate
NADPH = reduced nicotinamide adenine dinucleotide phosphate

Figure 1. The pentose phosphate pathway of the red cell glycolysis. The chemical reactions catalyse by G6PD and related chemical reaction.

G6PD deficiency is an inherited condition in which the activity of red cell G6PD enzyme is markedly diminished. The genes controlling G6PD structure and synthesis are located on the X chromosome, thus becoming subject to the unique phenomenon of X chromosome inactivation, sex-linked fashion. Hence it is fully expressed in affected males (hemizygotes). Because one X chromosome is inactivated during early development (Lyon hypothesis), female heterozygotes have two populations of red cells, deficient and normal. the inactivation process is random, and the total level of enzyme in the blood of female carriers (heterozygotes) varies markedly, ranging from normal to almost as low as that found in hemizygous males. There are more than 100 million affected individuals in the world population.[1]

Abnormality of pentose phosphate pathway and of glutathione metabolism give rise to haemolytic syndromes that have in common compromised generation of reduced glutathione (GSH) and, as a result, oxidative denaturation of haemoglobin and other erythrocyte proteins. Haemolytic crises often are initiated or intensified by certain drugs,[2-5] infections with malaria,[6-7] typhoid,[8] pneumonia,[9] viral hepatitis,[10,11] influenza A virus infection,[12] and other factors extrinsic to the red cell. According to this haemolytic problem, laboratory diagnosis of G6PD deficiency is therefore necessary.

There are several routine screening methods used in the determination of G6PD deficiency, for instance: heinz body test,[13] brilliant cresyl blue test,[14] methemoglobin reduction test,[15,16] dichlorophenol indophenol decolorization (DCIP) test,[17] phenazine methosulfate screening test[18] and fluorescent spot test.[19,20] These methods can easily detect the deficiency in hemizygous male and homozygous females, but the diagnosis of partial enzyme deficiency (female heterozygotes) is difficult because their blood contains varying proportions of normal and enzyme deficient erythrocytes.[21,22] Most of these screening methods need large amounts of blood samples, long duration of testing and the results need to be confirmed with the quantitative test.

The aims of this report are firstly to show that the fluorescent spot test introduced here is the appropriate technology for mass screening of G6PD deficiency in hospitals or health centres in the provinces or in field work. Secondly to compare the diagnostic results of the fluorescent spot test and the quantitative test (comparative method).

Materials and Methods

Blood collection

Samples were obtained from 205 patients at the Haematology Unit, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University. The peroid of collecting the samples was 14 months. There were 134 males and 71 females. Blood were collected in acid citrate dextrose (ACD) (0.25 ml of ACD containing 1.3 % trisodium citrate, 0.5% citric acid and 1.5% dextrose per 1.0 ml whole blood)

Blood samples can be kept at 4°C about 1-2 weeks before testing with the fluorescent spot test.[20] Only fresh blood is required for quantitative test (enzyme assay).

Determination of G6PD deficiency

1. Fluorescent spot test

The spot test principle is based upon the hydrolysis reaction of G6PD in the pentose phosphate pathway (figure 1), which results in the reduced NADP (NADPH) which fluoresces

under long wave ultra violet light, by using UV lamp model UVL-21, Blak-Ray Lamp, Ultra Violet Products, Inc. San Gabrial, California, USA.

This screening method was carried out in accordance with that of Beutler[19,20] and with some modifications by decreasing the volume of blood samples and the test reagents five times. However the concentrations of the chemicals used are equivalent. The technique of soaking the filter paper (Whatman No 1) in saturated ammonium sulfate solution[23] is still performed, and results in considerably intensified fluoresced NADPH at the outer circle of the spots.

Preparation of the reaction mixture

G6PD	0.01 M	2 portions
NADP	0.0075 M	1 portion
GSSG	0.008 M	1 portion
Saponin	1%	2 portions
0.2 M sodium phosphate pH 7.4		4 portions

The reaction mixture can be kept at -20°c for two years.[20]

Preparation of filter paper The Whatman No 1, filter paper, first soaked in a saturated ammonium sulfate solution and then dried.

Test procedure Approximately 2 ul of sedimented red blood cell are added to 20 ul of reaction mixture. The blood-reagent mixture is allowed to stand at 25°c for 10 minutes. the mixture is then spotted on prepared filter paper, allow to dry about 10-15 minutes, and it is then examined visually under long wave UV light.

Normal samples fluoresce brightly, while deficient samples (hemizygotes) show less or no fluorescence. Sample from heterozygotes show an intermediate degree of fluorescence. (figure 2).

2. Quantitative test (Standard Method)[24,25]

Enzyme activity was quatitated by adding a certain amount of haemolysate to

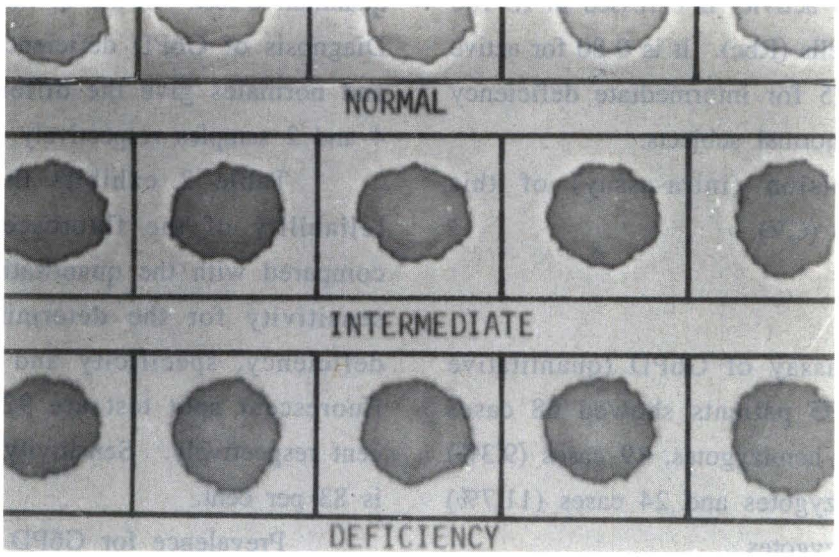


Figure 2. G6PD deficiency intermediate and normal under long wave U.V. light.

Table 1. G6PD deficiency test from 205 samples, comparison of the results between fluorescent spot test and quantitative test (Standard Method).

		QUANTITATIVE TEST		
		DEFICIENCY	INTERMEDIATE	NORMAL
FLUORESCENT SPOT TEST	DEFICIENCY	80	1	1
	INTERMEDIATE	4	20	1
	NORMAL	3	3	92

an assay mixture containing buffer, G6PD and NADP. The rates of NADPH generation was measured from 0-10 minutes at 340 nm. The details of preparation of haemolysate, test reagent and assay procedure have previously been described.[24,25]

The G6PD activity is exressed in IU/100 ml of red blood cells (Rbc). It is 0-80 for active deficiency, 90-175 for intermediate deficiency and 150-350 for normal subjects.

The precision (intra-assay) of this technique is 7.8% (CV).

Results

Enzyme assay of G6PD (quantitative method) from 205 patients showed 68 cases (33.1%) for male hemizygotes, 19 cases (9.3%) for female homozygotes and 24 cases (11.7%) for female heterozygotes.

Figure 2 shows the results of the fluorescent spot test under long wave ultra-violet light. control samples showed bright fluorescence while heterozygote (intermediates) showed medium degrees of fluorescence. Enzyme deficient samples show no fluorescence.

Table 1 shows the diagnostic results between the fluorescent spot test and the quantitative test. In use of the standard method. Diagnosis of G6PD deficiencies, intermediates and normales give the different results of 7, 4 and 2 samples respectively.

Table 2 exhibits the percentage of reliability of the fluorescent spot test, as compared with the quantitative method. The sensitivity for the determination of G6PD deficiency, specificity and accuracy of the fluorescent spot test are 92, 98 and 95 per cent respcetively. Sensitivity for intermediates is 83 per cent.

Prevalence for G6PD deficiencies were 42.4% and for intermediates were 11.7%

Table 2. Per cent reliability of fluorescent spot test, in comparison with quantitative method.

	Fluorescent Spot Test
Sensitivity :-	
Deficiency	92.0
Intermediate	83.3
Specificity	97.9
Accuracy	95.0
+ve Predictive Value	97.6
-ve Predictive Value	92.9
Post test likelihood if test negative	7.1

Prevalence : G6PD deficiency = 42.4%
Intermediate = 11.7%
Total number of samples = 205

Discussion

From our study the fluorescent spot test was found to be an appropriate screening method. It showed good reliability in screening programmes for male hemizygotes, female homozygotes and also female heterozygotes (intermediates).

The female heterozygotes are difficult to detect by general screening methods since varying proportions of normal and deficient red cells coexist. According to the lyonization of the proportion of the X chromosomes bearing the G6PD deficient gene and normal G6PD gene. Thus G6PD of heterozygotes will vary from normal to abnormal.[21,22,26]

The modified fluorescent spot test presented here gives a better diagnosis of intermediates (female heterozygotes) by the addition of oxidized glutathione to the test reagent,[20] using phosphate buffer pH 7.4[19] and soaking the filter paper with saturated ammonium sulfate.[23] After decreasing the test reagent and blood samples by 5 times from usual, the test still showed good reliability with 83% and 92% sensitivity for G6PD intermediate and deficiency, respectively, when compared to the standard method. In particularly, the sensitivity for intermediates was higher than Solem et al 13%,[23] with a

similar specificity of 98-99%.

However, false positive and false negative results were detected. These were caused by either a temperature-dependent enzyme inactivation or by the quenching effect of haemoglobin.[27] Long time blood keeping can also cause incorrect results due to auto-haemolysis. The proteolytic enzyme from haemolysed red blood cells can destroy G6PD, and the normal G6PD level may be changed to intermediate or deficiency.

The false negative results might be caused by time-dependent accumulation of NADPH. The incubation time was set to 10 minutes.[28] Even the reduced NADP occurring in the hydrolysis of glucose is constant from 5-20 minutes.[29] The incubation time of 10 minutes choosen here is also adequate for assaing 30-40 samples at the same time.

In acute haemolytic crisis of the A⁻ variety, the high reticulocyte count may produce a normal result of G6PD[1] which will reduce the sensitivity of the method. To get the true result sometimes it is necessary to centrifuge the blood sample and use only mature rbc which sediment at the bottom of the tube.[30,31]

Conclusion

The advantages of the modified fluorescent spot test introduced here are high specificity, only small samples are needed, short assay time, inexpensive, simple, able to use semiquantitatively and the test reagent being kept stable for 2 years.

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