ORIGINAL ARTICLE

Detection of Partial G6PD Deficiency using OSMMR2000-D Kit with Hb Normalization

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ABSTRAK

Penyakit kekurangan glucose-6-phosphate-dehyrogenase (G6PD) merupakan penyakit kekurangan enzim paling lazim di dunia termasuklah Malaysia. Saringan kekurangan separa enzim ini menggunakan darah tali pusat adalah sangat penting memandangkan mereka juga berisiko untuk mendapat hemolisis akut. Dalam kajian ini, kadar prevalens bagi kekurangan separa G6PD ditentukan dengan menggunakan kit ujian OSMMR-D dimana ianya mengintegrasikan kaedah pernormalan hemoglobin dan kadarnya dibandingkan dengan kaedah ujian titik pendafluoran (FST). Sejumlah 236 subjek dari kalangan kanak-kanak, berumur di antara 1 bulan hingga 12 tahun dan; 614 bayi-bayi perempuan telah dipilih untuk kajian ini. Penentuan julat normal aktiviti G6PD dan; paras penentu untuk kekurangan separa dan sepenuhnya ditentukan mengikut WHO Working Group (1989). Kadar prevalens untuk kekurangan separa untuk kedua-dua kumpulan pesakit (perempuan) dibuat menggunakan kit esei enzim ini dan keputusannya dibandingkan dengan FST. Dalam kajian ini, didapati 15.7% (18/115) kanak-kanak perempuan diklasifikasikan kekurangan separa G6PD menerusi kaedah ujian esei enzim (aktiviti G6PD: 4.23-5.26U/gHb), tetapi FST hanya dapat mengesan 0.9% (1/115) yang mempunyai aktiviti G6PD minimal. Kadar prevalens kekurangan separa G6PD pada bayi-bayi perempuan adalah 3.42% (21/614) menggunakan ujian esei enzim berbanding 0.49% (3/614) menggunakan FST. Kesimpulannya, hasil kajian ini membutikan bahawa ujian FST gagal mengesan pesakit kekurangan separa G6PD (heterozigot). Sehubungan dengan itu, kami menyarankan penggunaan

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ujian kuantitatif esei G6PD dengan OSMMR-D kit memandangkan ianya lebih sensitif untuk mengesan penyakit kekurangan G6PD dalam bayi-bayi perempuan berbanding dengan FST.

Kata kunci: separa, kekurangan G6PD, enzim, esei, FST

ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzyme deficiency worldwide including Malaysia. Screening of cord blood for partial G6PD deficiency is important as they are also prone to develop acute haemolysis. In this study, we determined the prevalence of partial G6PD deficient in paediatric population aged 1 month-12 years and normal term female neonates using OSMMR-D kit with haemoglobin (Hb) normalization and compare it with florescence spot test (FST). A total of 236 children, aged between between 1 month-12 years and 614 normal term female neonates were recruited for this study. Determination of normal means for G6PD activity and; cut-off points for partial and severe deficiency were determined according to WHO Working Group (1989). Determination of prevalence for partial deficiency for both groups (female patient) was done using this enzyme assay kit and findings were compared with FST. In this study, 15.7% (18/115) female children were classified as partial G6PD deficient by quantitative enzyme method (G6PD activity: 4.23-5.26U/gHb). However, FST only detected 0.9% (1/115) with minimal G6PD activity. The prevalence of partial G6PD deficiency in female neonate group was 3.42% (21/614) by enzyme assay versus 0.49% (3/614) by FST. This study concluded that our routine screening method using FST was unable to diagnose female heterozygotes. We recommend using this quantitative enzyme assay method by OSMMR-D kit since it was more sensitive in detecting G6PD deficiency in female neonates compared to FST.

Keywords: partial, G6PD deficiency, enzymes, assay, FST

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the common cause of neonatal jaundice in Malaysia. Neonatal screening for G6PD deficiency in Malaysia using the FST has long been established since 1980. The fallback of screening G6PD deficiency by this method is that semiquantitative test is not reliable in detecting female heterozygotes whereby they may have enzyme deficiency ranging from 20% to 60% of the normal residual activity. Detection of partially deficient G6PD individuals is important as they too are prone to develop acute hemolysis (Luzzatto & Mehta 1995).

In a study conducted by Reclos et al. (2000) involving 2000 neonates, it was found that FST was able to detect all male neonates with severe G6PD deficiency in which the G6PD activity

was less than 2.1 U/gHb (less than 20% of the normal residual activity). However, FST misclassified almost all cases with G6PD enzyme activity between 20- 60% of the normal residual which was interpreted as normal FST. These cases were found to be exclusively in females. These findings were in concordance with a local study by Ainoon et al. (2003) involving 976 neonates. The study demonstrated that 46.3% of the cases with partial G6PD deficiency were misclassified as normal by FST with enzyme activity ranging from 2.92 to 8.7 U/gHb. They were found to be exclusively females, as well. Both studies emphasized on the importance of correct classification and proposed the employment of fully quantitative method as a screening tool, in which the partially G6PD deficient patients are also at risk of developing acute haemolytic crises similar to their male counterparts.

The OSMMR2000-D G6PD assay is a quantitative enzyme assay. This kit offers rapid, fully automated, quantitative measurement of G6PD coupled to a simultaneous evaluation of the haemoglobin (Hb) content in the sample, expressing the results in Units/gram haemoglobin (U/gHb). Hb normalization will allow for correction of all samples against a selected standard or control used in the test by 'normalizing' all analyte values so that they correspond to the same quantity of sample (Azma et al. 2010) had established the normal reference range for G6PD enzyme activity level in Malaysian neonates and adults using this kit. The overall mean value for neonate and adult population were 12.43±2.28 U/gHb and 9.21 U/gHb respectively. The normal reference range for neonate population was 10.15-14.71 U/gHb while the adult population was lower, between 6.61-11.81 U/gHb. In this study, there were no significant differences in mean G6PD activity between males and females for Chinese and Malays. This lack of difference in red cell G6PD activity level among ethnic and gender groups has been reported previously in Malays and Chinese in Malaysia using Sigma kit (Ainoon et al. 2003). Measurement of G6PD activity using OSMMR-D kit is slightly more expensive and laborious compare to FST. However, this test is far more sensitive in detecting partial G6PD individuals (Azma et al. 2010).

In this study, we established the normal range of G6PD activity using OSMMR-D kit with haemoglobin (Hb) normalization for paediatric population age one month to 12 years old. We also determined the prevalence of partial G6PD deficient in this population and normal term female neonates using similar enzyme assay kit and compared it with FST.

MATERIALS AND METHODS

A total of 236 subjects from paediatric population aged between 1 month-12 years, who received treatment at Universiti Kebangsaan Malaysia Medical Centre (UKMMC) and 614 term female neonates born in UKMMC were enrolled in this study. The patients' peripheral blood samples and cord blood in EDTA tube were sent to the Haematology Unit, Department of Diagnostic Laboratory Services,

UKMMC.

For the establishment of normal range, selected paediatric blood samples were tested for full blood count using fully automated haematology analyzer (Coulter®GENS). Those with normal haemoglobin level were included in this study.

For FST, samples were applied to Whatman's filter paper and dried at room temperature. The test was carried out according to the method described by Beutler and Mitchell (1968). The spot was examined for fluorescence production from reduced pyridine nucleotide (NADPH) under a long-wave ultraviolet light source. Those with normal FST were included in the determination of normal range for G6PD activity and cut-off points for partial G6PD deficiency. Those showed minimal G6PD activity with FST were included in the prevalence of partial G6PD deficiency study.

For the quantitation of G6PD activity, we used the OSMMR2000-D G6PD assay kit with haemoglobin normalization from R&D Diagnostics (Holargos, Greece). The measurement of enzyme activity was done using a spectrophotometer (Ultramicroplate Reader EL808, Bio Tek, Instruments). Method for quantitation of G6PD activity using this assay kit has been described in a previous study (Azma et al. 2010).

Normal probability plots for variables of normal paediatric samples in each subgroup by age, sex and race were carried out to ascertain that the distribution was normal. Analysis of variance for the level of G6PD enzyme activity by sex, ethnic origin and age groups was carried out. This was followed by comparison of means in G6PD assay values between sex, ethnic origin and different age groups. A result of $p<0.05$ was considered as statistically significant. Then the means, SD, ranges of G6PD activity for this paediatric group were determined. A 20% and 60% cut-off point for the diagnosis of severe and partial G6PD deficiency respectively, were also established in this group (WHO Working Group 1989).

For female neonates group, their cord blood samples were sent for FST analysis and quantitation of G6PD activity by OSMMR-D G6PD assay with Hb normalization. Prevalence of partial G6PD deficiency was determined in both paediatric and female neonatal group and findings were compared between the two methods.

RESULTS

NORMAL MEAN OF G6PD ACTIVITY IN CHILDREN AGED 1 MONTH TO 12 YEARS OLD

From 236 paediatric subjects, aged between 1 month-12 years, only 214 subjects were included for the determination of normal reference range for G6PD activity. The other 22 subjects had absent or minimal enzyme activity by FST and G6PD enzyme assay showed G6PD activity less than 6.5U/gHb. They were considered as outliers and were excluded from the analysis. Out of 214 subjects, 117 were males (54.7%) and 97 were females (45.3%). Majority of the subjects recruited were Malays with 66.1% (n=156), followed

by Chinese 18% (n=43), Indians 2.5% (n=6) and others 3.8% (n=9). There were no significant differences in the mean normal G6PD activity among age groups (Table 1). There were also no significant differences in the mean normal G6PD activity among races (Table 2), and among gender in each racial group (Table 3).

The overall mean value for normal G6PD activity was 10.18±3.36 U/gHb (2SD). The reference range for normal G6PD activity established from this population was 6.82-13.54 U/gHb. The upper and lower limit cut-off points for partial G6PD deficiency were 6.11 U/gHb (60% of the normal mean) and 2.03 U/gHb (20% of normal mean), respectively (as recommended by WHO Working Group (1989). The upper limit for total/severe deficiency was 2.03 U/gHb (20% of the normal mean G6PD activity for paediatric population). The intrabatch and interbatch coefficient of variance for samples with normal G6PD enzyme activity level was 4.6%.

PREVALENCE OF PARTIAL G6PD DEFICIENCY IN CHILDREN AGED 1 MONTH TO 12 YEARS OLD IN UKMMC

The analysis for prevalence of partial G6PD deficiency by FST and enzyme assay was based on 236 samples. The results of screening for partial G6PD deficiency by FST and OSMMR-D kit were shown in Table 4. In male paediatric group, none of them were found to be G6PD partially deficient either by FST or enzyme assay. There was one out of 115 cases (0.9%) in female paediatric group which showed minimal enzyme activity by FST, while 18 cases (15.7%) were identified as partial G6PD deficiency by quantitative enzyme assay. Therefore, only one case of partial G6PD deficiency was detected by routine FST and was later confirmed with enzyme assay. The other 17 (14.8%) cases which had normal FST were not detected by this method. These children with partial G6PD deficiency had enzyme activity level ranged from 4.23-5.26 U/gHb.

PREVALENCE OF PARTIAL G6PD DEFICIENCY AMONG FEMALE NEONATES BORN IN UKMMC

A total of 614 term female neonates (459 Malays, 124 Chinese, 13 Indians and 18 others) were enrolled in this study. The overall mean normal G6PD activity and cut-off points for partial G6PD deficiency used in this study were based on study done by Azma et al. (2010).

For this study, 21 (3.42%) female neonates were found to have G6PD activity between 20-60% of the normal mean (2.03U/gHb-6.12U/gHb) by OSMMR-D kit and three cases were diagnosed by FST method (Table 5).

Racial breakdown showed that 2.28% (14 cases) were Malays, 0.98% (6 cases) were Chinese and 0.16% (one case) were Indian neonates diagnosed by OSMMR-D kit (Table 6). In the female Malay neonates group, only two (0.44%) cases were interpreted as minimal on FST while 12 other cases (2.61%) were normal by this method. All 14 (3.05%) cases of partial G6PD deficiency including two cases diagnosed by FST method were readily detected by OSMMR-D kit. The G6PD enzyme activity levels ranged from

Table 1: G6PD activity levels in different age groups in children age 1 month-12 years old

Age group	No. of cases	Mean G6PD activity (U/gHb)	Upper limit of total deficiency (20% of mean value)	Upper limit of partial deficiency (60% of mean value)
1 month-1 year	24	9.64±3.34	1.93	5.78
1-2 year	37	10.85±2.94	2.17	6.51
3-4 year	34	10.55±3.18	2.11	6.33
5-6 year	24	9.73±3.76	1.95	5.84
7-8 year	33	10.05±2.42	2.01	6.03
9-10 year	26	9.85±3.60	1.97	5.91
11-12 year	36	10.17±3.84	2.03	6.10
Total	214	10.18±3.34	2.04	6.11

There were no significant differences in the mean G6PD activity among different age groups in children age 1 month to 12 years old ($p>0.05$).

Table 2: G6PD activity levels in different ethnic group for children age 1 month-12 years

Ethnic group	No. of cases	Mean G6PD activity (U/gHb)	Upper limit of total deficiency (20% of mean value) U/gHb	Upper limit of partial deficiency (60% of mean value) U/gHb
Malay	156	10.28±3.22	2.05	6.14
Chinese	44	10.07±3.68	2.01	6.04
Indians	6	9.01±3.26	1.81	5.45
Others	8	9.81±3.98	1.96	5.89
Total	214	10.18±3.36	2.04	6.12

There were no significant differences in mean normal G6PD activity between various racial groups ($p>0.05$).

Table 3: G6PD activity levels for normal male and female paediatric group aged 1 month-12 years old

Races	Gender	No. of cases	Mean G6PD activity (U/gHb)	Upper limit of total deficiency (20% of mean value) U/gHb	Upper limit of partial deficiency (60% of mean value) U/gHb
Malay	Male	86	10.40±3.38	2.08	6.24
	Female	70	10.12±3.00	2.02	6.07
Chinese	Male	23	10.01±3.5	2.00	6.01
	Female	21	10.14±3.92	2.03	6.08
Indians	Male	3	8.73±2.94	1.75	5.24
	Female	3	9.28±4.10	1.86	5.57
Others	Male	5	9.12±2.44	1.82	5.47
	Female	3	10.95±2.80	2.19	6.57
Total	Male	117	10.23±3.42	2.05	6.14
	Female	97	10.13±3.30	2.03	6.08

There were no significant differences in the mean G6PD activity between males and females in each racial group ($p>0.05$)

Table 4: Detection of partial G6PD deficiency in paediatric group aged 1 month-12 year by FST and enzyme assay

Gender	Method	No. cases with total deficiency (G6PD activity <20%)	No. cases with partial deficiency (G6PD activity 20-60% normal)	No. cases with normal G6PD activity	Partial G6PD deficiency individuals (%)
Male (n=117)	FST	4	0	117	0
	G6PD enzyme assay	4	0	117	0
Female (n=115)	FST	0	1*	114	0.9
	G6PD enzyme assay	0	18#	97	15.7

*One case of partial G6PD deficiency detected by FST was interpreted as minimal G6PD activity and was confirmed by enzyme assay method.
#The range for partial G6PD deficiency in this paediatric population were between 2.03U/gHb - 6.12U/gHb.

Table 5: Detection of partial G6PD deficiency in female neonates by fluorescent spot test and enzyme assay methods.

	Method	No. of cases with partial deficiency (G6PD activity 20-60%)	No. of cases with normal G6PD activity	Partial G6PD deficient individuals (%)
Female (n=614)	FST	3*	611	0.49
	Enzyme assay	21	593	3.42

*The three cases of partial G6PD deficiency detected by FST were interpreted as minimal G6PD activity and confirmed by enzyme assay.

Table 6: G6PD activity in partial G6PD deficiency and overall frequency according to ethnic group, by fluorescent spot test and enzyme assay methods.

Ethnic group	No. (frequency) diagnosed by fluorescent spot test	No. (frequency) diagnosed by enzyme method	Partial G6PD deficiency (%)
Malay (n=459)	2 (0.44%)	14 (3.05%)	3.05
Chinese (n=124)	1 (0.81%)	6 (4.84%)	4.84
Indians (n=13)	0	1 (7.69%)	7.69
Others (n=18)	0	0	0
Total (n=614)	3 (0.49%)	21 (3.42%)	3.42

3.17-7.37 U/gHb.

Meanwhile, only one (0.81%) case was interpreted as minimal by FST in the Chinese female neonates as compared to all six cases (4.84%) diagnosed by enzyme assay method. The G6PD activity levels for this group range from 4.02-7.10 U/gHb. The female Indian neonate with partial G6PD deficiency was only detected by OSMMR-D kit and not diagnosed by FST method. There were no partial G6PD deficient neonates detected in other ethnic groups.

Therefore, the overall frequencies of partial G6PD deficiency detected by FST and OSMMR-D kit were 0.49%

and 3.42%, respectively.

DISCUSSION

Quantitative enzyme assay however is not routinely available in most hospitals in Malaysia. In our Haematology Laboratory, we have had introduced quantitative enzyme assay using OSMMR2000-D kit employing the Hb normalization technique and normal range for G6PD activity for neonates and adults in our laboratory using this kit have been established (Azma et al. 2010). In this study, we established the normal range, the mean and the standard deviations for G6PD activity for paediatric population age 1 month to 12 years old.

The overall mean value for paediatric population age 1 month-12 years old was 10.18 3.36 U/gHb. Comparing the mean normal value of G6PD activity for neonates and adults (12.43 U/gHb and 9.20 U/gHb, respectively) which have been established earlier, (Azma et al. 2010) the mean normal value of G6PD activity in this paediatric population age 1 month-12 years old falls between these two values. This was probably due to presence of circulating young red cells which was known to be higher in neonates and gradually decreased as they get older. The young red cells (reticulocytes) contained higher G6PD activity than mature red cells. Apart from that, contamination with high total white cells during hemolysate preparation in neonates may contribute to higher G6PD activity level in this population.

We also compared the mean values for G6PD activity among different age groups in this paediatric population and there was no significant differences ($p>0.05$). As this study recruited paediatric population age between one month till 12 years old, therefore variation in the haemoglobin level among various groups need to be considered as well. In normal physiology, immediately after birth, the rates of Hb synthesis and red cell production fall fairly steeply to a minimum level by the second month of life, in response to the sudden increase in the tissue oxygenation at birth. In our setting, term babies have mean Hb value of 15g/dL at four weeks and 12.4g/dL by eight weeks and slowly reduced to about 12.1g/dL by 6-8 month of life. However, there is a slight dip in the mean Hb noticed after 6 to 12 month with a mean Hb of 11.6g/dL but later the level sustained between 11 – 13g/dL. The reticulocytes count fall after birth as erythropoeisis is suppressed and increases to normal values by 6-8 weeks of age. With the rise in 2,3-DPG level and fall in Hb F level in the first few month of life, the haemoglobin-oxygen dissociation curve gradually shifts to the right, which lead to increase in delivery of oxygen to the tissue and hence, ameliorating the effect of falling Hb. Therefore, the mean Hb level in paediatric population between age 1 month and 12 years old varies between 11.6-15.0g/dL. As compared to the neonate population, the Hb and reticulocytes level is higher and therefore the mean for G6PD enzyme level is expected to be higher in this group. This normal physiological fluctuation in Hb levels defines the need for a method that can 'normalize'

the Hb level so that the level of G6PD enzyme level will not be affected.

The mean normal for G6PD activity between male and female children in various age groups also showed no significant differences ($p>0.05$). Similar findings also seen in neonates and adult population in a study done by Azma et al. (2010) where it was shown that gender did not affect the G6PD enzyme activity level in their respective population.

The comparison of mean normal for G6PD enzyme activity in various ethnic groups showed no significant differences ($p>0.05$). There were also no significant differences in the mean normal G6PD activity between male and female in various ethnic groups ($p>0.05$). Similar findings have been described by Azma et al. (2010) in neonates and adult populations, however they only compared two major ethnic groups in Malaysia, namely Malays and Chinese. Ainoon et al. (2003) has also described similar findings by using the Randox G6PD kit. In this study, the cut-off point for G6PD deficiency in paediatric population was 6.12 U/gHb and the range for partial G6PD deficiency was 2.04-6.12 U/gHb. The range for partial G6PD deficiency for neonate and adult population by Azma et al. (2010) were 2.5-7.4 U/gHb and 1.84-5.52 U/gHb, respectively. Therefore the range for partial G6PD deficiency established for the paediatric population from this study was expected to fall in between the neonate and adult ranges.

Prevalence of partial G6PD deficiency detected by both FST and G6PD enzyme assays were determined in all 236 subjects. None of the male children (121) had partial G6PD deficient. However there were four male children with severe G6PD deficiency detected by both methods. Their G6PD activity level was <2.04 U/gHb.

In the female children group, one out of 115 cases (0.9%) had minimal G6PD activity by FST and was confirmed partial G6PD deficiency by quantitative G6PD enzyme assay. Meanwhile, the quantitative G6PD assay was able to pick up 18 cases (15.7%) with G6PD deficiency including the sample detected by FST. Therefore, the FST misdiagnosed 14.8% subjects as normal. The residual G6PD activity ranged from 41.6%-51.7% (4.23-5.26 U/gHb). These cases were classified as partial G6PD deficiency. A study by Wolf et al. (1987) revealed that sensitivity of FST for detection of partial G6PD deficiency was low (32%) as compared to detection of total male G6PD deficient.

In this study, we found that the overall prevalence of G6PD deficiency by FST was 2.12% (5/236), but by using quantitative enzyme assay the prevalence was 9.32%. Therefore, semiquantitative screening test failed to detect children with partial G6PD deficiency but no problems in detecting those with severe G6PD deficiency. These findings were supported by previous study that conducted in neonate population in Hospital Kuala Lumpur which showed that semiquantitative method was able to detect neonate with severe G6PD deficiency if G6PD activity was less than 20% of normal mean (Boo et al.

1994). Therefore, screening with FST was able to diagnose male neonates with severe G6PD deficiency.

In this study we also looked at the prevalence of partial G6PD deficiency in 614 female neonates born in UKMMC. The cut-off points for partial G6PD in this population were based on a study done by Azma et al. (2010). The established range for partial G6PD deficiency for this population was 2.5-7.4U/gHb. Subjects with enzyme activity within this range were classified as partial G6PD deficiency. From 614 subjects, 21 (3.42%) had G6PD activity level between 20 - 60% of the normal mean by enzyme assay method and only three cases showed minimal G6PD activity by FST.

The prevalence of partial G6PD in female neonates by using FST and quantitative enzyme assay were 0.49% and 3.42% respectively. Therefore, these indicate that 2.93% (18) cases were missed by routine screening test and these cases were found to be partial G6PD deficiency. These findings were supported by a study by Ainoon et al. (2003) involving 976 neonates (both male and female neonates), they found that the overall prevalence by using FST and quantitative enzyme assay were 3.28% and 7.17%, respectively, which conclude that semiquantitative screening method failed to diagnose 3.9% of neonates with G6PD deficiency, and all were female. Another study by Reclos et al. (2000) recruiting 2150 neonates revealed that 24 subjects classified as normal by FST found to be partially deficient by the quantitative enzyme method and they too were all female. The rate of detection was much higher by enzyme method and had good sensitivity and specificity whereby all cases detected by FST were readily diagnosed by this quantitative method. Meanwhile FST tend to give negative result for mild to moderate deficiencies. This could also possibly explain the misdiagnosis of 44% of infants with severe hyperbilirubinaemia in Malaysia (Selvaraju 1999).

In conclusion, the OSMMR-D G6PD kit is excellent in detecting partial G6PD deficiency in female population. Since, the FST does not appear to miss G6PD deficient males, it is proposed that for financial reasons, quantitative screening could be limited to female neonates. The test is easy to perform, less laborious, more sensitive, rapid produce results with affordable cost.

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