Abstract

Objective: To screen newborn population for G6PD deficiency and compare results of the Dye Reduction Test (DRT) with Quantitative estimation of G6PD deficiency.

Study Design: Cross-sectional study at Haematology Department, Army Medical College, National University of Sciences and Technology and Military Hospital, Rawalpindi, (January-August, 2011)

Methodology: Cord blood (2.5 ml blood in K3EDTA bottle) samples were obtained from 120 newborns after informed consent from parents. 65 patients were males and 55, females. Samples were evaluated for G6PD deficiency using qualitative (DRT) and quantitative spectrophotometric technique. Data obtained was analyzed using SPSS Windows version 17.

Results: Frequency of G6PD deficient cases was 2.5% by DRT and 4.2% by quantitative estimation. DRT misclassified 2 cases of partial deficiency as normal in female population.

Conclusion: DRT has a diagnostic accuracy of 98.3%, is specific, cost effective and easy to perform. However, heterozygote female detection continues to be a challenge with this technique.

Keywords: Glucose 6 Phosphate Dehydrogenase, Dye Reduction Test.

Introduction

Glucose 6 phosphate dehydrogenase (G6PD) is an important enzyme. In the red blood cell (RBC), it maintains the erythrocyte pool of reduced glutathione (GSH), thus protecting the erythrocyte from oxidative stress. Deficiency of this enzyme affects almost 400 million people. It prevalence is 3.6-28.0 percent in Africa, 6.0-15.8 percent in Asia, 3.0-29.0 percent in the Mideast and 62 percent in Kurdish Jews. Studies conducted in Pakistan have estimated the prevalence for G6PD deficiency to be 1.8-3.8 percent. Khan et al conducted a study in 2000 on 800 subjects and found the frequency to be 3.5 percent in Punjabis and 8.3 percent in Pathans. Studies in Lahore and Peshawar put the prevalence at 4-14 percent in jaundiced neonates.

As the G6PD gene is located on the X chromosome, the clinical manifestations are usually confined to hemizygous men but ‘female carriers can be clinically affected due to lyonisation’. During embryonic development in females, one of the X chromosomes is genetically inactivated. In G6PD deficient female heterozygotes, two different populations of red blood cells (normal and G6PD deficient) result in expression of intermediate G6PD activity levels. Due to this, some of such patients are misclassified as normal by most diagnostic techniques. Today, we know that analysis of genomic DNA in leucocytes is the
most accurate technique for diagnosing G6PD deficiency\textsuperscript{14}. This study was undertaken to screen newborn population for G6PD deficiency and compare the dye reduction test (DRT) with the spectrophotometric (quantitative) estimation of G6PD as diagnostic modalities. This study provides insight to the frequency of enzyme deficiency in the newborn population in a country where most population surveys have been adult-based. Kernicterus associated mortality in Pakistan ranges from 2 - 4.3 percent\textsuperscript{15}. It also highlights an important aspect of diagnosis, namely, the detection of female heterozygote for the enzymopathy. Furthermore, as Pakistan lies in the malaria endemic zone, prior knowledge of patient G6PD status also helps the clinician in prescribing antimalarials judiciously, particularly primaquine.

**Methodology**

A cross-sectional study was conducted in Hematology Department, Army Medical College, National University of Sciences and Technology and Military Hospital Rawalpindi from January to August 2011. Non probability convenient sampling technique was used and 120 cases were evaluated (after informed consent from parents). The study was approved by institutional ethical review committee, and subjects were enrolled after taking informed consents from their parents. The families of the enrolled individuals were provided information related to G6PD deficiency. All patients were newborns (males and females) and cord blood was the sample evaluated. Babies delivered preterm and patients having reticulocyte count greater than 6 percent were not considered for the study. Old samples (not evaluated within 6 hours of sampling) were also excluded from the study.

For each evaluation, 2.5 ml of cord blood was taken in K3 EDTA (Ethylene diamine tetra acetic acid) bottle (manufactured in Italy) and gently mixed. All samples were analysed within 6 hours of collection. Samples were stored in the refrigerator at 4° C prior to analysis. Prior to testing for G6PD, complete blood count (CBC), evaluations of peripheral film (using Leishman stain) and reticulocyte count (using Brilliant Cresyl Blue stain) were performed on every sample. Sysmex Hematology Analyzer KX-21 was used for CBC. The Hb concentration (g/dl) was used in subsequent calculations. Evaluation of RBC morphology was done to rule out abnormally raised reticulocyte count (greater than 6 percent) or any other pathology.

For the qualitative G6PD analysis, the kit (Product Code No.GSX25936A) manufactured by Span Diagnostics, France was used. The principle is based on the reduction of blue dye dichlorophenol indophenol (DCPIP) by NADPH, manifested as a color change (from blue to red). The time for this to occur is 30-60 minutes in normal subjects. The time for deficient subjects is 140 minutes to 24 hours. For the quantitative analysis, the kit manufactured by AMP Diagnostics, Austria (BD6400-E V3.0 CE) was used. The reduction of NADP to NADPH in the reaction mixture is recorded by a spectrophotometer at 340nm, in kinetic mode \[16\]. For this purpose, Microlab-200 (Merck, Netherlands) was used. Absorbance of reaction mixture was recorded at 5 minutes interval (denoted as \(A_1\) and \(A_2\)). Test was performed as per manufacturer’s instructions and G6PD activity was computed in U/g Hb.

According to WHO criteria, total enzyme deficiency is defined as 10 percent of normal G6PD activity. Partial deficiency is defined as 10–60 percent of normal enzyme activity. Based on this, the lower and upper cut off points for partial deficiency were calculated. All data was analysed through the statistical packages for social sciences (SPSS), Windows version 17. Frequency of G6PD deficient cases diagnosed by either method (DRT
and quantitative) was calculated. Mean and standard deviations were calculated for quantitative variables. 2 sample t-test was used to calculate the p value. At 95% confidence level, p-value less than 0.05 was taken as significant. Diagnostic accuracy, sensitivity, specificity, positive predictive value and negative predictive value were calculated with the help of following statistical 2x2 table.

<table>
<thead>
<tr>
<th>DRT</th>
<th>No of G6PD Deficient cases</th>
<th>No of cases with Normal G6PD activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of G6PD Deficient Cases</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>No of cases with Normal G6PD activity</td>
<td>c</td>
<td>d</td>
</tr>
</tbody>
</table>

\[a = \text{true positives}, \ b = \text{false positives}, \ c = \text{false negative}, \ d = \text{true negatives}\]

Sensitivity: \[\frac{a \times 100}{a + c}\]
Specificity: \[\frac{d \times 100}{d + b}\]
Positive Predictive Value: \[\frac{a \times 100}{a + b}\]
Negative Predictive Value: \[\frac{d \times 100}{c + d}\]
Diagnostic Accuracy: \[\frac{(a + d) \times 100}{a+d+b+c}\]

**Results**

A total 120 newborns conforming to the set parameters of inclusion and exclusion criterion, were included in this study. Out of 120 patients, 65 (54.17%) were male and 55 (45.83%), female. Mean normal (± standard deviation) G6PD activity for the 65 male newborns was 16.42U/g Hb ± 4.04. Mean normal (± standard deviation) G6PD activity for the 55 female newborns was 16.31U/g Hb ± 3.68. The p value was calculated by using 2 tailed student t test and it was noted that there was no significant difference (p value = 0.8) in this value between sexes. The collective mean value for 120 newborns was thus 16.37 U/g Hb ± 2.76.

Based on WHO criteria (mentioned in methodology section), the lower and upper cutoff points for partial deficiency were calculated as 1.64 U/g Hb and 9.82 U/g Hb respectively. These results are summarized in Table 1. Calculations revealed that the range for severe enzyme deficiency in the population under study was 0-1.29 U/g Hb. Patients with partial G6PD deficiency had enzyme activity in range of 4.33-8.48U/g Hb. Normal G6PD activity had a range of 10.85-21.85 U/g Hb.

In the male population, G6PD deficiency was diagnosed in 3.1 percent of cases by both screening tests. These results are summarized in Table 2. Upon quantitative estimation of G6PD activity, it was noted that all these cases had severe enzyme deficiency with G6PD activity in range of 0 - 1.29

**Table 1: Mean normal G6PD activity and values of upper and lower limits for partial G6PD deficiency as per WHO criteria (expressed in U/g Hb)**

<table>
<thead>
<tr>
<th>Mean normal G6PD activity (U/g Hb)</th>
<th>Upper limit of total deficiency (10% of mean normal U/g Hb)</th>
<th>Upper limit of mild/partial deficiency (60% of mean normal U/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.37</td>
<td>1.64</td>
<td>9.82</td>
</tr>
</tbody>
</table>

**Table 2: Number of cases with partial and severe G6PD deficiency in newborn male population, with DRT and Quantitative G6PD estimation**

<table>
<thead>
<tr>
<th></th>
<th>Method</th>
<th>No of cases with partial deficiency</th>
<th>No of cases with severe deficiency</th>
<th>No of cases with normal G6PD activity</th>
<th>G6PD deficient individuals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males (n = 65)</td>
<td>DRT</td>
<td>0</td>
<td>2</td>
<td>63</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Quantitative</td>
<td>0</td>
<td>2</td>
<td>63</td>
<td>3.1</td>
</tr>
</tbody>
</table>
U/g Hb.
In the female population, 1.8 percent cases were diagnosed as deficient by DRT opposed to 5.5 percent by enzyme assay. This means that 3.7 percent of deficient females were misclassified as normal by the DRT. These results are summarized in Table 3. Incidentally all these cases had a range of 4.33 – 8.48 U/g Hb, as determined by quantitative G6PD estimation. The net frequency of G6PD deficiency in the newborn population as diagnosed by DRT and quantitative estimation is 2.5 percent and 4.2 percent respectively. Out of the 120 cases analyzed, 5 were diagnosed as deficient by enzyme assay. On the other hand DRT could only detect 3 cases (Table 4). When analyzed further, it was noted that out of the 3 G6PD deficient cases diagnosed with DRT, 2 had severe enzyme deficiency and 1 had partial enzyme deficiency. The DRT could not detect the other 2 cases of partial enzyme deficiency (due to phenomena of X chromosome inactivation, as discussed in succeeding text) that were by confirmed by quantitative estimation of G6PD. The statistical 2x2 table (Table 4) and formulae mentioned in methodology section were used to calculate various statistical parameters for the DRT. Sensitivity was found to be 60 percent. Specificity and positive predictive value were 100 percent. Negative predictive value was 98.3 percent. Diagnostic accuracy was 98.3 percent.

**Discussion**
Our study was conducted on 120 neonates and 4.2 percent diagnosed to be G6PD deficient. Two newborns were diagnosed with severe enzyme deficiency, while 3 had partial enzyme deficiency. It is noteworthy that the DRT accurately diagnosed cases of severe enzyme deficiency but failed to diagnose cases of partial enzyme deficiency, except one (as confirmed by quantitative estimation). Moreover, these patients were females. This observation is consistent with the fact that most female heterozygotes are misclassified as normal with most screening tests. This is attributed to the concept of X chromosome inactivation12.

A similar study was conducted in Malaysia16 in 2003 on 976 neonates. The fluorescent spot test (FST)17 classified 3.28 percent of the population as G6PD deficient whereas the enzyme assay estimated the prevalence to be 7.17 percent. This meant that 3.9 percent of enzyme deficient neonates had been missed by the fluorescent spot test. Similar observations were the result of an-

| Table 3: Number of cases diagnosed with partial and severe G6PD deficiency in newborn female population, with DRT and Quantitative G6PD estimation |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Method          | No of cases with partial deficiency | No of cases with severe deficiency | G6PD deficient individuals (%) |
| Females (n = 55) | DRT             | 1                           | 45                          | 1.8 |
|                 | Quantitative    | 3                           | 52                          | 5.5 |

<table>
<thead>
<tr>
<th>Table 4: Statistical 2x2 table showing comparison of normal and G6PD deficient cases as diagnosed by qualitative (DRT) and quantitative methods</th>
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<tr>
<td><strong>Qualitative Analysis</strong></td>
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<tr>
<td>No of G6PD deficient cases</td>
</tr>
<tr>
<td>No of cases with normal G6PD activity</td>
</tr>
<tr>
<td>Sensitivity = 60%</td>
</tr>
</tbody>
</table>
other study in 2000, employing the FST to screen 2000 neonates\textsuperscript{18}. 2.4 percent of neonates with partial deficiency were misclassified as normal and they were all females. Severely deficient cases (enzyme activity less than 10 percent of normal) were accurately diagnosed with FST. According to another study, the FST has 32 percent sensitivity in heterozygote female detection\textsuperscript{19}. It must be borne in mind that the ICSH recommends the FST as the most reliable screening modality for G6PD deficiency\textsuperscript{20}. In addition to these observations, we also calculated values for normal activity in newborns are higher than that of adults. Ainoon and his colleagues calculated a value of 14.8 U/g Hb\textsuperscript{16}. In our study, mean normal G6PD activity for newborns was calculated to be 16.37 ± 2.76 U/g Hb. The range for normal G6PD activity was 10.85-21.85 U/g Hb.

The WHO recommends the FST, most recent studies have employed various dye reduction tests. The DCPIP reduction test is one of the most commonly used techniques in our country. It is cost effective and easy to perform. It does not require any sophisticated equipment unlike the FST which requires ultraviolet lamp. A limitation of this technique is its inability to accurately diagnose cases of partial enzyme deficiency. As already discussed, numerous studies have found the FST to be lacking in the same area.

The role of DRT as a screening tool has already been established in many hospitals/laboratories of the country. This is very cost effective when compared to the cost of hospitalization, exchange transfusions and treating complication of kernicterus in newborns with G6PD deficiency. Furthermore, the test is easy to perform and does not require special equipment like the requirement of ultraviolet lamp in fluorescent spot test.

Another aspect which deserves special attention is the testing of G6PD status of patients before prescribing certain drugs, especially antimalarial, which can precipitate hemolysis in such individuals. A recent study revealed that the most common G6PD variant in Pakistan is G6PD Mediterranean 563C-T, which is characterized by very low enzyme activity\textsuperscript{21}. Bearing in mind the endemicity of malaria in our country, this is a valid public health issue\textsuperscript{22}. The antimalarial Primaquine is essential for radical cure of malaria owing to its gametacydal properties but its use remains limited in Pakistan. Since G6PD testing is not routinely carried out here, physicians are very cautious and hesitant about prescribing primaquine to the population in general owing to its hemolytic potential. Such a practice has created serious impediments in the eradication of malaria and has also resulted in frequent cases of relapse in our community.

Measures to increase the utility of DRT will benefit the community as opposed to taking no steps at all in this direction. However, the physician must exercise caution when females are being screened. Quantitative tests for estimation of G6PD activity may be used in such scenarios. Established enzyme deficient cases in the family, unexplained jaundice/anemia in the patient or in a sibling or family history of splenomegaly/cholelithiasis should impel the physician to suspect G6PD deficiency in the newborn female\textsuperscript{23}. Furthermore, judicious G6PD testing must also be utilized as a malaria treatment and control tool in our efforts to effectively combat and eradicate malaria.

**Conclusion**

1. This study estimates the frequency of G6PD deficiency in the newborn population to 2.5 percent by DRT and 4.2 percent by quantitative estimation. The DRT accurately identifies all cases of severe G6PD deficiency but misclassifies some cases of heterozygote females with partial G6PD deficiency as normal.
2. The range of G6PD activity determined by quantitative estimation in this study is in agreement with the values calculated by other researchers\textsuperscript{18}.

This study has revealed that the DRT has a diagnostic accuracy of 98.3%. Heterozygote female detection continues to be a challenge with this technique but this holds true for alternative screening techniques as well. In light of our observations, we believe that the role of the DRT as screening test of choice in our setup needs to be assessed further and where resources and facilities permit, the quantitative test for G6PD estimation should also be employed.

**Competing interests:** None declared

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