Original Article

INTRODUCTION

All living organisms, be yeasts or protozoa, plants or animals express the glucose-6-phosphate dehydrogenase (G6PD) enzyme, [1] a “housekeeping” enzyme [2]. Complete absence of the enzyme is unknown in the human species [2]. G6PD is essential to maintain stability of red blood cells by protecting them from oxidative damage [3].

G6PD deficiency is the most common erythroenzymopathy [4] affecting 10% of the world population with a frequency of 0-27% in India. Children with G6PD can develop acute haemolytic crises precipitated by particular foods or drugs causing significant morbidity. As primary prevention is not available, the only way to avoid morbidity is to recognize such children early in life and prevent exposure to triggering agents and drugs.

Aims/ Objectives: 1. To screen and determine the frequency of G6PD in cases of neonatal hyperbilirubinemia (> 12.9 mg/dl).

2. To assess the usefulness of methaemoglobin reduction (MR) test in neonatal screening for G6PD.

METHODOLOGY

The study was conducted in the departments of pathology and paediatrics at Kempegowda Institute of Medical sciences Hospital and Research centre, Bengaluru, Karnataka. This observational study included 310 neonates with hyperbilirubinemia (> 12.9 mg/dl) ranging in age from birth to 28 days. The male: female ratio was 1.3:1, with 80.3% of neonates presenting between 3-5 days post-birth with jaundice. Majority of the neonates were from non-consanguineous marriages (91.6%). Birth weight of 83.7% of the neonates was found to be > 2.5 kgs.

Frequency of G6PD was 1% (3 females) by MR test. All the G6PD deficient neonates (3/310 cases) were females (P=0.045), from non-consanguineous marriages.

Conclusion: Frequency of G6PD in neonatal hyperbilirubinemia was 1% (3/310 cases) suggesting low prevalence in south India. MR test appeared to be a good screening test as it was effective in picking up heterozygotes (all three were females, P=0.045).

Keywords: G-6PD, Hyperbilirubinemia, Methaemoglobin reduction (MR) test, Neonates

ABSTRACT

Glucose-6-Phosphate Dehydrogenase deficiency (G6PDD), is one of the commonest X-linked inherited erythroenzymopathy affecting 10% of the world population with a frequency of 0-27% in India. Children with G6PDD can develop acute haemolytic crises precipitated by particular foods or drugs causing significant morbidity. As primary prevention is not available, the only way to avoid morbidity is to recognize such children early in life and prevent exposure to triggering agents and drugs.

Aims/ Objectives: 1. To screen and determine the frequency of G6PDD in cases of neonatal hyperbilirubinemia (> 12.9 mg/dl).

2. To assess the usefulness of methaemoglobin reduction (MR) test in neonatal screening for G6PDD.
Karnataka. This study population consisted of 310 neonates (birth to 28 days) who had hyperbilirubinemia and were admitted in the NICU. The inclusion criteria were all term (>36 weeks gestational age) neonates with hyperbilirubinemia (total serum bilirubin > 12.9 mg/dl). Neonates with total serum bilirubin < 12.9 mg/dl and premature neonates (less than 36 weeks of gestation) with hyperbilirubinemia were excluded from the study.

The relevant personal history was obtained from the hospital records as per the proforma.

MATERIALS

0.5 ml of venous blood was collected into a tube containing EDTA (1mg/1ml).

All cases were screened for G6PD deficiency by Methaemoglobin Reduction Method

METHAEMOGLOBIN REDUCTION TEST

Principle: In this reaction, Sodium Nitrite converts haemoglobin to methaemoglobin. When no methylene blue is added methaemoglobin persists but, incubation of samples with Methylene Blue allows the stimulation of the pentose phosphate pathway in subjects with normal G6PD levels. The methaemoglobin is then reduced during the incubation period to haemoglobin. In G6PD deficient subjects, the block in the pentose phosphate pathway prevented this reduction and the methaemoglobin remained.

Reagents

1) Sodium Nitrite – [S15729, Batch No. 102070, Nice Chemicals]
2) Dextrose – [G10229, Batch No. 007633, Nice Chemicals]
3) Methylene blue – [C.I.52015, Product no. 39692, Batch no. L953, Glaxo laboratories]

Solution 1 - Sodium nitrite - 180 mmol/L - 1.25 g
Dextrose - 280 mmol/L - 5 g

Stock Solution A - Dissolve 5 g of dextrose and 1.25 g of sodium nitrite in 100 ml of distilled water. This solution has a shelf life of 1-2 months.

Solution 2 - Methylene blue - 0.4 mmol/L - 150 mg
<table>
<thead>
<tr>
<th>Age in days</th>
<th>Total number of patients</th>
<th>Number of patients with G6PD</th>
<th>Incidence</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D-2D</td>
<td>21</td>
<td>0</td>
<td>0.0</td>
<td>0.707</td>
</tr>
<tr>
<td>3D-5D</td>
<td>249</td>
<td>2</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>6D-10D</td>
<td>38</td>
<td>1</td>
<td>2.63</td>
<td></td>
</tr>
<tr>
<td>&gt;10D</td>
<td>2</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gender</th>
<th>Total number of patients</th>
<th>Number of patients with G6PD</th>
<th>Incidence</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>177</td>
<td>0</td>
<td>0.0</td>
<td>0.045*</td>
</tr>
<tr>
<td>Female</td>
<td>133</td>
<td>3</td>
<td>2.25</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Birth weight(kg)</th>
<th>Total number of patients</th>
<th>Number of patients with G6PD</th>
<th>Incidence</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2.5</td>
<td>50</td>
<td>1</td>
<td>2.0</td>
<td>0.698</td>
</tr>
<tr>
<td>2.5-3.5</td>
<td>251</td>
<td>2</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>&gt;3.5</td>
<td>9</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mode of delivery</th>
<th>Total number of patients</th>
<th>Number of patients with G6PD</th>
<th>Incidence</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>130</td>
<td>3</td>
<td>2.31</td>
<td>0.241</td>
</tr>
<tr>
<td>Forceps</td>
<td>7</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Vacuum</td>
<td>16</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>LSCS</td>
<td>157</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Consanguineous Marriage</th>
<th>Total number of patients</th>
<th>Number of patients with G6PD</th>
<th>Incidence</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>284</td>
<td>3</td>
<td>1.05</td>
<td>0.598</td>
</tr>
<tr>
<td>Yes</td>
<td>26</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total Serum Bilirubin (mg/dl)</th>
<th>Total number of patients</th>
<th>Number of patients with G6PD</th>
<th>Incidence</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;15.0</td>
<td>186</td>
<td>2</td>
<td>1.1</td>
<td>0.148</td>
</tr>
<tr>
<td>15.0-20.0</td>
<td>102</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>20.0-25.0</td>
<td>17</td>
<td>1</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>&gt;25.0</td>
<td>5</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>310</td>
<td>3</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>

Stock Solution B - Dissolve 150 mg of methylene blue in 1 L of distilled water. This solution has a shelf life of one year.

Procedure
1) Three test tubes were taken and labelled as test, positive control and negative control respectively.
2) To all the three test tubes 0.5 ml of blood was added.
3) The reagents were then added accordingly to all three test tubes as given below:
   a) To the test tube labeled ‘Test’, 0.025 ml of the stock solution A and 0.025 ml of the stock solution B were added.
   b) To the test tube labeled ‘Positive control’, only 0.025 ml of the stock solution A was added.
   c) To the test tube labeled ‘Negative control’, no reagents were added.
4) All the three test tubes were then incubated at 37°C for 3 hrs in a water bath.
5) After incubation another three separate test tubes were taken and 10 ml of distilled water was added to each tube. These test tubes were also labelled Test, Positive control and Negative control respectively.
6) Then, 0.1 ml (100 µl) of the incubated mixture was added to its respective test tubes with distilled water and the colour in each of the different tubes were then compared.

Interpretation of the test: [Table/Fig-1,2]
Positive Control - Brown colour
Deficient cases, while among the 133 female neonates was not statistically significant. (P=0.0707). There were no G6PD deficient neonates presenting with jaundice < 2 days or > 10 days after birth, however one case was G6PD deficient, with an incidence of 2.63. There were no G6PD deficient neonates presenting with jaundice < 2 days or > 10 days after birth, however was not statistically significant. (P=0.0707).

Among the 177 male neonates, there were no G6PD deficient cases, while among the 133 female neonates there were three G6PD deficient cases, with an incidence of 2.25 among females (P=0.045).

Of the 50 neonates < 2.5 kg, one case showed G6PD deficiency, while the remaining 2 cases were seen among the 251 neonates having a birth weight of 2.5-3.5 kg, giving an incidence of 2.0 and 0.79, respectively. No correlation was seen between birth weight and G6PD deficiency (P=0.698). There was no statistical significance between the mode of delivery and G6PD deficiency (P=0.241).

The history of consanguinity was absent in 284 neonates, among which all three of our G6PD deficient neonates were seen, giving an incidence of 1.05. Thus, there was no correlation seen.

**DISCUSSION**

North India [3,8,9,10] showed a comparatively higher frequency of G6PD deficiency among neonates, while South India [11,12] recorded a lower frequency. Higher prevalence is reported from north and west India than Southern India [13] [Table/Fig-6]. Prevalence of G6PD deficiency in Indian community was first reported from the Parsi population of Mumbai in the year 1963 by Baxi et al., [14].

Correlation of G6PD deficiency with religion has not shown statistical significance [15]. Similarly in the present study 91.9 % of the neonates were Hindus with only a few (8.1%) Muslims. All 3 of the G6PD deficient neonates were Hindus. We could not correlate the frequency with various sub-castes due to non-availability of records. However, one study form Bangalore [15] showed a frequency of 7.8 %, but in this study there was a higher degree of consanguinity (41.1 %) when compared to our study with consanguinity of 8.4 %.

Onset of jaundice in neonates with G6PD deficiency has been studied by many authors, [3,16-19] who showed that none of the G6PD deficient neonates presented with jaundice during the first 24 hours of life and most of them manifested with jaundice between the 2nd to 4th day [3,17]. Sialo C L et al., [18] showed the mean age of onset of jaundice to be 2.65 days among G6PD deficient neonates and 2.99 days among G6PD normal neonates. All three of our G6PD deficient neonates showed onset of jaundice between 3 to 6 days after birth with a mean of 4.7 days. This is in accordance with most of the studies.

Some authors have screened all neonates, irrespective of the presence or absence of hyperbilirubinemia for G6PD deficiency with varying percentages of prevalence [Table/Fig-7]. Various authors have used different cut-off values for hyperbilirubinemia [Table/Fig-8]. The frequency of G6PD being 1% in our study. Looking at the [Table/Fig-3,4], we are of the opinion that we may have missed G6PD deficient neonates who had total serum bilirubin levels of less than 13 mg/dl. However our study is in concordance with the studies by Ramadevi AR and Naushad SM [11] (0.045 %) and Ramadevi AR (0.2 %) [12], both from South India.

Screening for G6PD deficiency can be done using the

**STATISTICAL METHODS**

Descriptive and inferential statistical analysis has been carried out in the present study. Results on continuous measurements are presented on Mean ,SD (Min-Max) and results on categorical measurements are presented in Number (%). Significance is assessed at 5 % level of significance.

Chi-square/ Fisher Exact test has been used to find the significance of study parameters on categorical scale between two or more groups.

**Statistical software:** The Statistical software namely SAS 9.2, SPSS 15.0, Stata 10.1, MedCalc 9.0.1, Systat 12.0 and R environment ver.2.11.1 were used for the analysis of the data and Microsoft word and Excel have been used to generate graphs, tables etc.

**RESULTS**

This was an observational study to screen and determine the frequency of G6PD deficiency in cases of neonatal hyperbilirubinemia (> 12.9 mg/dl) along with assessment of the usefulness of MR test in neonatal screening for G6PD deficiency.

A total of 310 neonates were screened for G6PD deficiency, among which only 3 (1%) tested positive [Table/Fig-3]. The study population comprised of 177 (57.1%) males and 133 (42.9%) females with a male:female ratio of 1.3:1. Majority (91.9%) of the neonates were Hindus with only a few (8.1%) Muslims. There were no Christians / other religion in the study population. Blood group O was the commonest with 43% belonging to O+. [Table/Fig-4]. A serum total serum bilirubin level of 13-15 mg/dl was seen in majority (72.1%) of the neonates, while only 5.7% had levels more than 20 mg/dl.

Majority (80.3%) of the neonates in the study population developed jaundice between 3-5 days of age, followed by 12.3% between 5-10 days and about 0.6% after >10days post birth.

Association of Frequency of G6PD Deficiency with clinical variables: [Table/Fig-5]

Of the 249 neonates presenting with jaundice between 3-5 days after birth, two were G6PD deficient with an incidence of 0.80, while among the 38 neonates presenting with jaundice 6-10 days after birth, one case was G6PD deficient, with an incidence of 2.63. There were no G6PD deficient neonates presenting with jaundice < 2 days or > 10 days after birth, however was not statistically significant. (P=0.0707)

Among the 177 male neonates, there were no G6PD deficient cases, while among the 133 female neonates, among which all three of our G6PD deficient neonates were seen, giving an incidence of 1.05. Thus, there was no correlation seen.

**Negative Control**
- Clear red
**G6PD Normal subjects**
- Clear red
**G6PD Deficient subjects (males and females)**
- Brown colour
**G6PD Intermediate females**
- Colour varies between red and brown
Suvitha Thilakarajan et al., G-6PD Deficiency Screening in Neonatal Hyperbilirubinemia

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Serum bilirubin levels for inclusion criteria</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Madan N [8] et al.</td>
<td>1989</td>
<td>≥ 12 mg/dl</td>
<td>35.1 %</td>
</tr>
<tr>
<td>Al-Omran A [16] et al.</td>
<td>1999</td>
<td>&gt; 9 mg/dl</td>
<td>30.3 %</td>
</tr>
<tr>
<td>Atay E [17] et al.</td>
<td>2006</td>
<td>not known</td>
<td>3.8 %</td>
</tr>
<tr>
<td>Eghbalian F and Monsef AR [9]</td>
<td>2007</td>
<td>&gt; 12 mg/dl in term neonates</td>
<td>4.4 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt; 10 mg/dl in preterm neonates</td>
<td></td>
</tr>
<tr>
<td>Marzban A &amp; Mosavinasab N [27]</td>
<td>2009</td>
<td>&gt; 15 mg/dl</td>
<td>5.8 %</td>
</tr>
<tr>
<td>Silao CL et al., [18]</td>
<td>2009</td>
<td>≥ 10 mg/dl</td>
<td>16.7 %</td>
</tr>
<tr>
<td>Hussain M et al., [19]</td>
<td>2010</td>
<td>&gt; 9 mg/dl</td>
<td>16 %</td>
</tr>
<tr>
<td>Mondal M et al., [3]</td>
<td>2012</td>
<td>clinically evident jaundice</td>
<td>13.6 %</td>
</tr>
<tr>
<td>Dholakia A et al., [13]</td>
<td>2012</td>
<td>&gt; 10 mg/dl</td>
<td>10.6 %</td>
</tr>
<tr>
<td>Our study</td>
<td>2012</td>
<td>&gt; 12.9 mg/dl</td>
<td>1 %</td>
</tr>
</tbody>
</table>

Table/Fig-8: Studies done for G6PD deficiency among Jaundiced Neonates

three methods – the MRT, the FST (fluorescent spot test), ascorbate cyanide test [8] and dye decolorisation test [8]. There is a difference of opinion among the authors on whether to use only a qualitative or quantitative method of testing for G6PD deficiency or whether to use a combination of both. There is also disagreement on which method of screening test should be used. Madan N et al., [8] and Ahmed P and Ahmad KN [21] showed that the MRT screening test may not be sensitive enough with the possibility of false-negative reactions occurring. Few studies [8,22] suggested FST to have a higher sensitivity and specificity when compared with other screening tests. However, Akanni EO [5] and Sharma M et al., [23] demonstrated that MRT was equally sensitive as FST.

Muzaffer MA [24] in 2005 screened 2922 newborns using the FST in Saudi Arabia. G6PD deficiency was seen in 50 neonates (2%). Prevalence for boys was 3% and for girls was 0.9%. Being an X-linked recessive disorder, the frequency of affected males is expected to be equal to the number of heterozygote females, but this study failed to produce this result. Therefore the sensitivity of the screening test might not be sufficient to detect all the heterozygote females. Minareci E et al., [22] screened 1604 people at random using a standardized home made FST and 35 people (2.2%) were found to be G6PD deficient. FST is said to have the highest validity and specificity in the diagnosis of severe G6PD deficiency for both homozygote males and females.

MRT, is a cost-effective test with reagents commonly available in-house and therefore was chosen in our study. We used only a single screening test-MRT without the simultaneous use of a quantitative enzyme assay allowing a possibility of having false negatives and thus having missed some cases. Is the low frequency (1%) of G6PD deficiency in our study due to low sensitivity for MRT as a screening test or whether the because of actual low incidence in South India? Hence efficacy of MRT as the only screening test for mass screening may be proven when MRT is compared with a simultaneous enzyme assay.

WHO recommends screening for G6PD deficiency in populations which show a prevalence of 3-5% in boys [2]. Our study with a frequency of 1%, suggests that mandatory screening for G6PD in cases of neonatal hyperbilirubinemia may not be needed. Possibly in the future, G6PD screening in large population of neonates with simultaneous use of multiple screening tests in conjunction with enzyme assay could give a more accurate frequency. This may help us in taking a decision at our hospital regarding the mandatory screening for G6PD deficiency jaundiced neonate.

CONCLUSION

- The frequency of G6PD deficiency in south India may be lower, highlighting the geographic variation.
- MR test appeared to be successful to pick up heterozygotes.
- Mandatory screening for G6PD deficiency in neonatal hyperbilirubinemia may not be needed at our hospital.

LIMITATIONS

Owing to the small sample size and use of a single screening test, studies including a large number of neonates with simultaneous use of enzyme assays are needed to confirm the findings from this study.

REFERENCES


