Original Article

Prevalence of 563 C – T& 131 C-G G6PD polymorphisms in term and late preterm neonates with significant unconjugated hyperbilirubinemia from a tertiary level care neonatal unit in Uttarakhand

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ABSTRACT

Objectives: This study aimed to estimate the frequency of Glucose-6-phosphate dehydrogenase (G6PD) Mediterranean variant (563 C->T) and G6PD Odisha (131 C->G) variants along with the prevalence of G6PD deficiency quantitatively. This study also estimated peak serum total bilirubin level and duration of phototherapy and correlated with the G6PD levels and G6PD mutations. **Methods:** All the consecutive term and late pre-term neonates of either gender with significant hyperbilirubinemia were enrolled after written informed consent and were screened for G6PD deficiency (quantitatively). These neonates were also evaluated for G6PD gene polymorphisms. Exclusion criteria: Neonates with congenital anomalies or conjugated hyperbilirubinemia and those who received treatment before enrollment were excluded from this study. **Results:** A total of 150 infants with hyperbilirubinemia were enrolled. Males were two-thirds. Median G6PD levels were 11.9 units/gm of Hb (Inter quartile range [IQR]: 9.4–16.2). The prevalence of G6PD deficiency in our study population was 24% among the whole population and 30.6% in male infants. As per the World Health Organization (WHO) classification, majority (94%) were in class IV, 5.3% (n=8) had WHO Class III. All the eight infants in Class III were males. Mean (SD) and median serum bilirubin levels were 16.7 (1.1) mg/dL. Median duration of phototherapy was 24 h. Gene polymorphisms were done in 119 neonates. G6PD Mediterranean mutations were seen in 6.7% and G6PD Orissa mutations were seen in 4.2% neonates. There was a good correlation between G6PD deficiency and serum total bilirubin, mean duration of phototherapy and gender. **Conclusion:** This study reported 24% prevalence of G6PD deficiency among the study population with 6.7% prevalence of G6PD Mediterranean mutations and 4.2% prevalence of G6PD Orissa mutations.

Key words: Glucose-6-phosphate dehydrogenase deficiency, Mediterranean, Neonatal jaundice, Orissa

Glucose-6-phosphate dehydrogenase (G6PD) is a key enzyme present in the cells that catalyzes the initial step of the hexose-monophosphate pathway, which produces Nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is essential for maintenance of the effective redox potential that helps to guard erythrocyte membranes against oxidative stress and injury [1-4].

Predominant clinical manifestations due to G6PD deficiency include neonatal jaundice and acute hemolytic anemia [1]. Certain drugs (primaquine), infections, or foods (fava beans) can precipitate this phenomenon. The hemolysis results due to the inability of G6PD-deficient erythrocytes to survive the oxidative damage produced either directly or indirectly by these provoking agents. The G6PD enzyme has over 200 odd different biochemical variants [2]. Many G6PD gene variants are named after locations from where they were first discovered; for instance – Canton, Mahidol, Kaiping, and Viangchang (which are more prevalent in Asian populations) and G6PD Mediterranean, Orissa, Kalyan-Kerala, Coimbra (more common in India) [2,4]. G6PD gene is located near the telomeric region of distal arm of chromosome X (Xq28) [5]. This gene consists of 12 introns and 13 exons and has 18.5 kb size.

G6PD deficiency is one of the most frequent enzyme deficiencies known, affecting millions of people worldwide. Although initially, it was seen in Africa, southern Europe, the Middle East, and Asia, due to migration of people and enhanced

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transport facilities across the countries [6-14], it has spread all over the world. The red blood cell (RBC) is at especially high risk for this oxidative damage, as, unlike other cells, the hexose monophosphate pathway is the only source of NADPH in RBCs. Oxidative damage can be exacerbated by the high concentrations of oxygen normally present in these cells. The net result being damage to the cell membrane and hemolysis. In the absence of extraneous triggers of hemolysis or additional icterogenic factors [15-20]. G6PD deficiency is the fifth common congenital defect across the world, and the most common erythrocyte enzymopathy with a global prevalence of 4.9% [11-20]. It is highly prevalent in tropical (Asia and Africa) and subtropical regions alongside Mediterranean and Middle East countries. The most common being single-nucleotide substitution (missense variants), followed by two or more substitutions, deletions, and intron affecting mutations.

The role of G6PD deficiency has been elucidated in protection against malaria due to hindered antioxidant defense in ring-staged parasitized erythrocytes. Countries having high malaria transmission are in good correlation with the prevalence of G6PD deficiency [20-25]. According to the World Health Organization (WHO) malaria policy advisory committee meeting report (October 2019), it was suggested that G6PD will be classified according to enzyme activity as shown in Table 1 [12].

Previously, Yoshida *et al*.'s proposed classification of G6PD, in the year 1971, also known as the "WHO Classification [18]. Class I: <10% of normal enzyme activity (Severe enzyme deficiency: Chronic non-spherocytic hemolytic anemia; Class: II: <10% of normal (severe); Class: III: 10–60% of normal: Moderate-to-mild (intermittent hemolysis); Class: IV: 60–100% of normal enzyme activity (mild-to-normal); and Class V: >150%, twice of normal (increased enzyme levels).

The prevalence of G6PD deficiency varies from 2.3 to 27% with an overall prevalence of 7.7% in different regions of India [20-25]. However, the incidence has been reported to be even higher in countries like Bahrain which was up to 40% [17]. The higher preponderance is reported in tropical and subtropical areas. G6PD Mediterranean variant (563 C->T) and G6PD Odisha (131 C->G) variants are frequently reported from North India [16]. These gene polymorphisms result in unstable RBC membrane and predispose for hemolysis. Studies have shown that Erythrocytic G6PD enzyme activity is markedly reduced in G6PD Med type (0.64–1.1 IU/g Hb), but was moderate in G6PD Orissa type (1.2–3.1 IU/g Hb). The polymorphic mutations affect amino acid residues throughout the enzyme and decrease the stability of the enzyme in the red cell, possibly by disturbing protein folding.

Table 1: Recent world health organization malaria policy advisory committee meeting report (12) Classification of G6PD activity [12]

G6PD activity	Indicator sign
25% or less activity	(–)
25–65% activity	(+/–)
65–150% normal activity	(+)
>150% activity	(++)

The severe mutations mostly affect residues at the dimer interface or those that interact with the structural NADP molecule.

Aim

The aim of the study was to estimate the frequency of G6PD Mediterranean variant (563 C->T) and G6PD Odisha (131 C->G) variants and prevalence of G6PD deficiency quantitatively.

METHODS

This prospective and observational study was conducted in a tertiary center in North India over a period of 4 years [December 2017 to December 2021]. All the consecutive term and late pre-term neonates of either gender with significant hyperbilirubinemia (Defined as serum total bilirubin levels (STB) ≥15 mg/dl in term and ≥12 mg/dl in pre-term neonates or levels requiring phototherapy/Exchange transfusion zone as per American Academy of Pediatrics [AAP] charts) [26] were enrolled after written informed consent and were screened for G6PD deficiency (quantitatively). These neonates were also evaluated for G6PD gene polymorphisms. The neonatal clinical details (birth weight, gestational age, age of onset of jaundice, mode of delivery, and maternal details) and laboratory results (hemoglobin levels, reticulocyte count, G6PD levels, and gene polymorphisms) were recorded in a pre-designed and pre-tested performa.

Exclusion Criteria

Neonates with congenital anomalies, conjugated hyperbilirubinemia, those with history of blood, or exchange transfusion and phototherapy were excluded from this study. Conjugated bilirubin fraction was estimated by Jendrassik and Grof method [27]. The highest value of STB was taken as the peak STB. The decision to start and stop phototherapy or do an exchange transfusion was as per unit protocol, which is a modification of the AAP, revised guidelines published in the year 2004 [26].

Statistical Analysis

The baseline demographic data of neonates with hyperbilirubinemia were represented. The categorical variables were presented as frequencies and percentages; and continuous variables were reported as mean ± standard deviation. Comparisons between infants with G6PD deficiency and without G6PD deficiency were done using χ^2 test for categorical variables and Student's *t* test for continuous variables. The data were analyzed using SPSS version 22 (SPSS Inc., Chicago, IL, USA).

Ethical Clearance

From Institute ethics committee: AIIMS/IEC/18/495: Dated: Deember 29, 2018. Patient enrollment started after ethical clearance.

RESULTS

During the study period, 171 infants were eligible for enrollment in the study. Out of them, 21 infants were excluded due to various exclusion criteria (conjugated hyperbilirubinemia: Four, congenital malformations: Six, underwent exchange transfusion before enrollment: Two, received IVIG before enrollment: Four, and parents refused consent: Five). Hence, a total of 150 infants with hyperbilirubinemia were enrolled. Out of them, males were two-thirds (n=98, 65.3%) as shown in Fig. 1.

Median age of onset of jaundice was 46.5 h. Median G6PD levels were 11.9 units/gm of Hb [IQR: 9.4–16.2], while mean (SD) was 12.4 (4.2). As the infants who were enrolled were <1 month old, G6PD cutoff of 8.7 U/g of hemoglobin was used as per previous studies to differentiate G6PD deficient (G6PD-D) and sufficient groups. About 24% (n=36) of infants had G6PD deficiency as shown in Fig. 2. Among these infants with G6PD deficiency, 83.3% were males (Fig. 3). It was higher in male infants (30.6%, n=30/98). As per the WHO classification, none had Class I or Class II. About 5.3% (n=8) had WHO Class III and majority (n=141, 94%) were in Class IV and only one infant (0.7%) was in Class V (Fig. 4). All the eight infants in Class III were males (Fig. 5).

Thirteen infants (8.7%) had hemoglobin values <10 g/dL. Among these infants, 61.5% had Class III levels. All infants with WHO Class III had hemoglobin levels <10 g/dL. Mean (SD) serum bilirubin levels were 16.7 (1.1) mg/dL and median levels were almost similar: 16.8 (IQR: 15.8–17.6] mg/dL. Mean (SD) duration of phototherapy was 27.8 (7.1) h and median duration of phototherapy was 24 h. Fig. 6 reveals QQ plot of G6PD levels. Detection and characterization of G6PD gene mutations: 119 neonates out of 150 irrespective of their G6PD screening status underwent mutational analysis for G6PD Mediterranean and G6PD Orissa mutation by the PCR-RFLP method. Eight neonates (6.7%) had heterozygous mutations for G6PD Mediterranean as shown in Fig. 7. Rest of them were wild homozygous. Similarly, 4.2% of neonates (n=5) were found to have G6PD Orissa mutation. Fig. 8 reveals G6PD Mediterranean mutation.

One sample t test revealed significant relation between mean G6PD levels and STB ($p<0.001$). Similarly, there was statistically significant correlation between mean STB and duration phototherapy ($p<0.001$) as shown in Fig. 9. The likelihood ratio between quantitative G6PD levels and STB levels was 44.6 ($p<0.033$). Lambda coefficient was 0.36 ($p<0.001$). There was a good correlation between G6PD deficiency and STB, mean duration of phototherapy and gender.

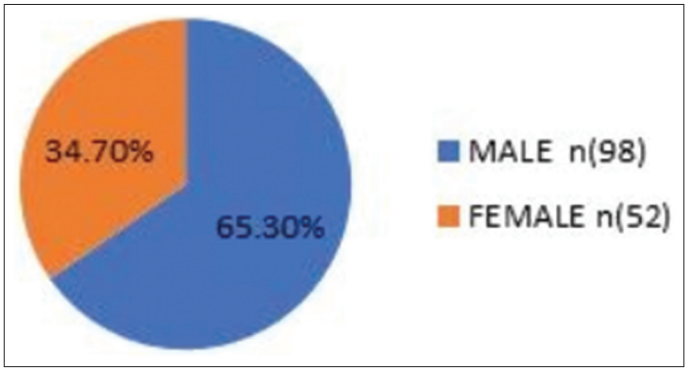

Figure 1: Gender-wise distribution of study population

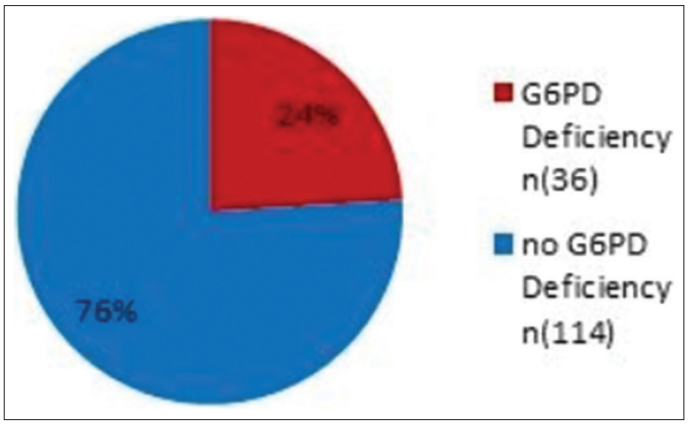

Figure 2: Prevalence of G6PD deficiency among population

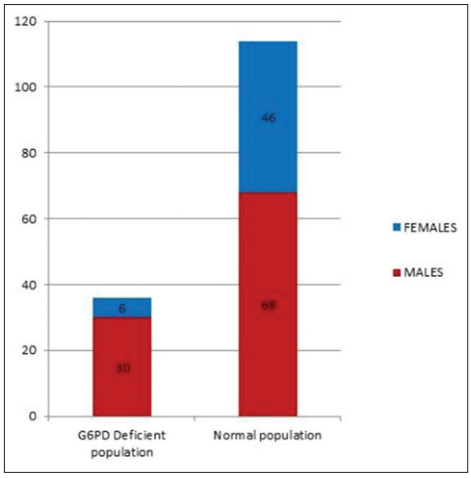

Figure 3: Gender-wise distribution of G6PD deficient and normal population

DISCUSSION

Our study is the first study in the state of Uttarakhand evaluating the G6PD deficiency status in infants attending to pediatric (and neonatal) Department. We have done quantitative estimation of G6PD levels followed by mutation analysis for both G6PD Mediterranean and Orissa mutations, which are common mutations seen in India [28-30]. The prevalence of G6PD deficiency in our study was 24% which was comparable to a previous study from North India by Agarwal *et al*. [25]. They have studied 77 neonates with hyperbilirubinemia. Of them, 19 babies had G6PD Mediterranean mutation (hemizygous males: 12, heterozygous females: Six, and homozygous female: One). Only two neonates had Orissa mutation (hemizygous male: One and heterozygous female: One). None of the controls had any G6PD mutation. The mutant *G6PD* gene was seen in 27.3%. All these 21 neonates with G6PD mutations had quantitative G6PD deficiency. In addition, three babies, who did not show the presence of G6PD Mediterranean or Orissa mutation, had quantitative G6PD

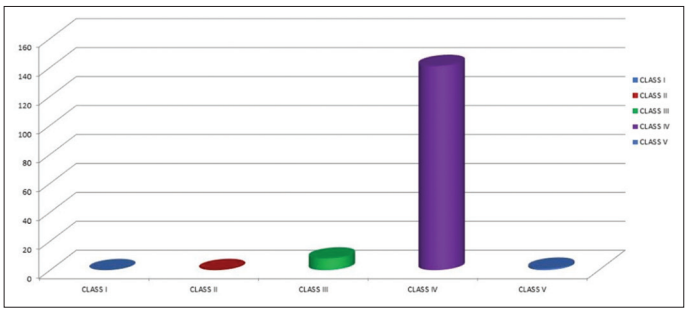

Figure 4: Distribution of study population as per the World Health Organization (WHO) criteria

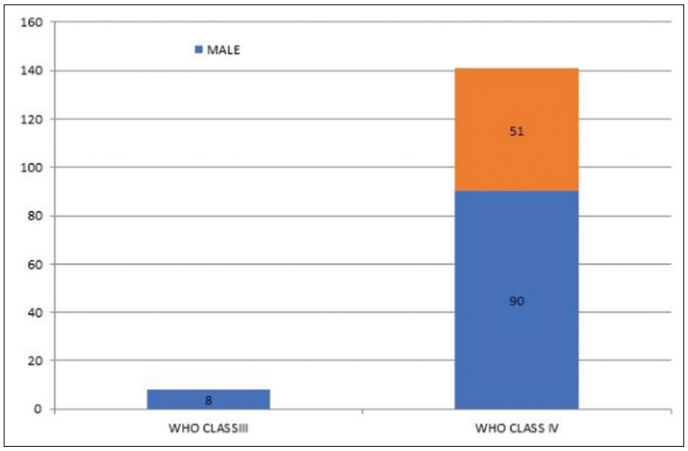

Figure: 5: Correlation of neonates with G6PD levels categorized in the WHO Class III and IV with gender [Class IV: Females: 51; Males: 90]

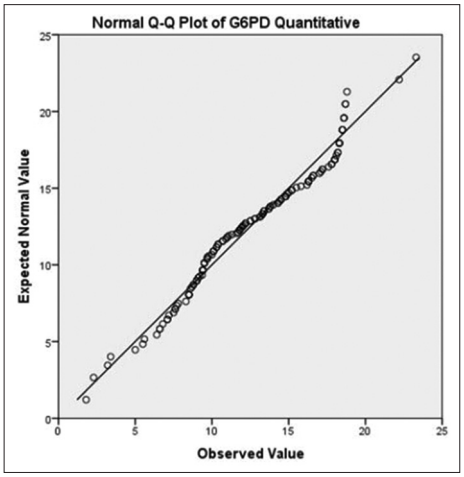

Figure 6: G6PD Quantitative estimation

activity -2SD of the normal and they were also labeled as G6PD-D for analysis. Hence, overall 24/77 neonates had g6PD deficiency (31%) which is very high. Furthermore, the predominant type of mutation was G6PD Mediterranean (24.7% of total population and 90.5% of overall mutations). Female neonates contributed to 38% of infants with mutation (8/21). In our study, we did not have controls due to difference in study design. We have enrolled all the infants with significant hyperbilirubinemia (STB>15 mg/dl).

In our study, quantitative G6PD estimation was done in all 150 infants. While 119 infants, only mutation analysis was possible. Thus, 79.3% of infants underwent mutation analysis. The prevalence of G6PD Mediterranean mutation was 6.7%, and G6PD Orissa mutation was 4.2%. Mediterranean mutation rate was lower than the study by Agarwal *et al*. Compared to their

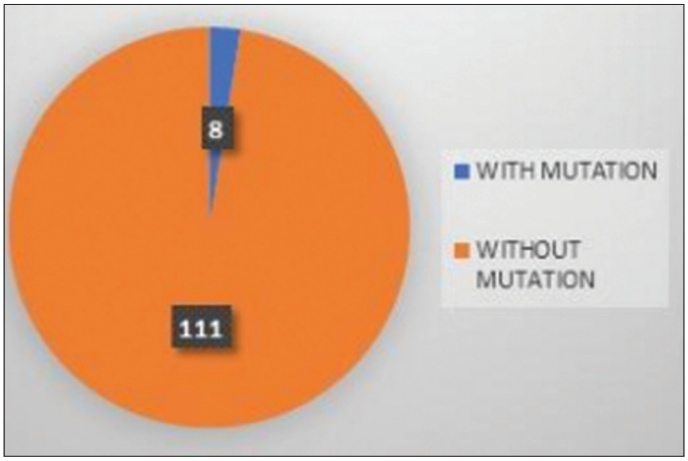

Figure 7: Prevalence of G6PD Mediterranean mutation

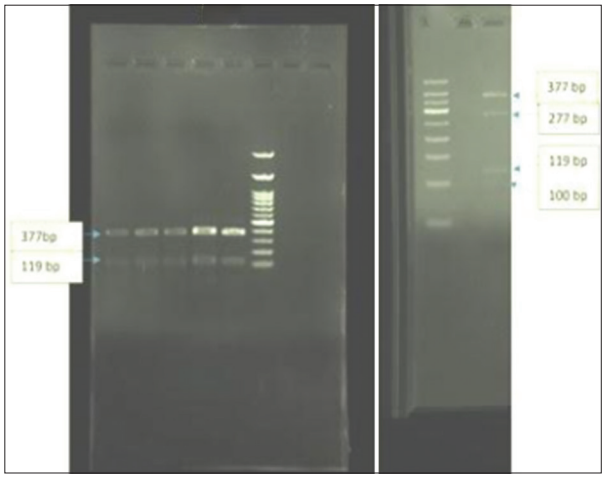

Figure: 8: RFLP: G6PD Mediterranean: Digested product after RFLP showing Wild normal (2 bands) and heterozygous mutant (4 bands) variants for Mediterranean G6PD gene

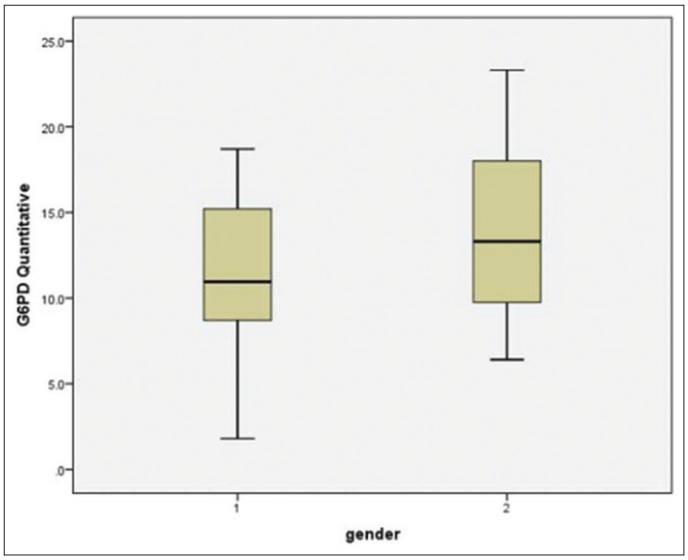

Figure 9: Correlation between G6PD level and gender

geography, our geographical region has lower prevalence of malaria which might be correlating with the lower mutation rate. However, the overall deficiency status was comparable to their study.

Cherepnalkovski *et al*. reported 7.4% prevalence of G6PD deficiency in their population from Southern Croatia, retrospectively [23]. Our study being prospective could detect more prevalence. They have used fluorescent spot test o screen 513 male children with neonatal hyperbilirubinemia.

Isa *et al*. conducted a retrospective case–control study from Bahrain to estimate G6PD deficiency among infants with neonatal indirect hyperbilirubinemia [31]. They also compared G6PD-deficient and G6PD-normal patients regarding hyperbilirubinemia and need for exchange transfusions (ET). They assessed risk factors for ET and kernicterus. Out of 1159 jaundiced neonates admitted, 1129 were included in the study, of whom 646 (57%) were male. They reported a very high prevalence of G6PD deficiency (42%), which is nearly 2 times that of ours. They also reported a higher prevalence of requirement for ET. In our study, none required ET and none had kernicterus.

Tanphaichitr *et al*. in their study from Thailand performed quantitative RBC G6PD assay in the cord blood of 505 male subjects [32]. They did case–control study. Their results showed 12% prevalence of RBC G6PD deficiency (61/505) which was half of our prevalence. However, we did not have a control group. They did not do mutation analysis.

Gibbs *et al*. from Jamaica found 69% G6PD deficiency in 23 neonates who had unexplained moderate or severe jaundice [33]. However, their sample size was small and they did not do mutation analysis.

CONCLUSIONS

A total of 150 infants with hyperbilirubinemia were enrolled. Out of them, males were two-thirds and females were one-third. Median G6PD levels were 11.9 units/g of Hb [(IQR):9.4–16.2], while mean (SD) was 12.4 (4.2). The prevalence of G6PD deficiency in our study population was 24% among the whole population and 30.6% in male infants. As per the WHO classification, majority (94%) were in Class IV and 5.3% (n=8) had WHO Class III. All the eight infants in Class III were males. Mean (SD) and median serum bilirubin levels were 16.7 (1.1) mg/dL. Median duration of phototherapy was 24 h. The prevalence of G6PD Mediterranean mutations was 6.7% and G6PD Orissa mutations were 4.2%. There was a good correlation between g6PD deficiency and STB, mean duration of phototherapy and gender. This study is first one to do mutation analysis and quantitative G6PD deficiency among neonates/infants with significant hyperbilirubinemia from the state of Uttarakhand.

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