Qualitative and quantitative assay of glucose 6 phosphate dehydrogenase in patients attending tertiary care center

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Research note

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Abstract

Objectives

The study was carried out to with the aim to find out the frequency of G6PD deficiency among the patients attending the hospital and to rationalize methemoglobin reduction test (Qualitative method) in reference to the spectrophotometric assay (Quantitative method). Timely screening of the patients for Glucose 6 phosphate dehydrogenase deficiency with appropriate screening method can play an important role in preventing hemolytic crisis that arises from therapeutic use of oxidative drugs like primaquine.

Result

The frequency of Glucose 6 phosphate dehydrogenase deficient cases was 3% by both of the employed tests. The mean ± SD of Glucose 6 phosphate dehydrogenase activity in the patients under study was 15.34 ± 4.7 IU/l in males, 16.01 ± 3.74 IU/l in females. G6PD activity was positively associated with reticulocyte count (r = 0.289, p-value = 0.004) and negatively with mean corpuscular hemoglobin concentration (r = -0.220, p-value = 0.028). The correlation of Red blood corpuscular count and Glucose 6 phosphate dehydrogenase was statistically significant (p-value = 0.048).

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is a rate limiting enzyme of the pentose phosphate pathway and is closely associated with the hemolytic disorders among patients receiving anti-malarial drugs, such as primaquine. [1] G6PD deficiency is an x-linked recessive hereditary disorder. [2] This disorder affects 200-400 million people worldwide. The G6PD gene codes for an enzyme that catalyzes the reaction that converts nicotinamide adenine dinucleotide phosphate ($NADP^+$) into NADPH which is a reduced form, in pentose phosphate pathway. [3]

G6PD deficiency has been classified into five classes according to the severity of the deficiency of the enzyme:

Class I deficiency is defined as severe deficiency and is associated with chronic non-spherocytic hemolytic anemia. Class II deficiency is also defined as severe deficiency with enzyme activity of 1% – 10% of normal activity. Class III deficiency are moderately deficient and their enzyme activity is 10%–60% of normal activity. Class IV and Class V individuals have normal and increased activity with an enzyme activity of 60%–150% and over 150%, respectively. [4]

At the public health level, at least simple screening methods for G6PD deficiency are needed in order to detect a hemizygous males and homozygous females to avoid an acute hemolytic crisis, newborn

hemizygous males and homozygous females to detect neonatal jaundice and early treatment and lastly, to detect heterozygous females, to give specific advice about the care of their newborn male infants. [5]

Not only primaquine but a large spectrum of drugs can induce hemolysis in G6PD deficient individuals. [6] Ingestion of fava beans (*Vicia Faba*) has been associated with hemolytic anemia in G6PD deficient individuals since a very ancient period of time. [7] Exposure of infant or mother to oxidant drugs or without exposure to such drugs leading neonatal jaundice due to G6PD deficiency can even lead to kernicterus. [8]

In chronic Non-Spherocytic Hemolytic Anemia, the affected individuals have a moderately severe hemolytic anemia in association with reduced activity of erythrocyte G6PD. [9]

For the identification of G6PD deficiency in patients, there are five types of phenotypic tests:

I. Direct enzyme activity assay: Spectrophotometry and Beutler's fluorescent spot test
II. Indirect assay: Methemoglobin reduction test and brilliant cresyl blue or formazan ring tests
III. Cytofluorometric assayHirono-1-methoxy PMS Sephadex method and WST8/1 methoxy PMS method
IV. Rapid point-of-care tests:Binax Now G6PD and Carestart G6PD [10,11]

Screening of the population is desirable in the regions with a G6PD deficiency prevalence ≥ 3–5% before the use of contraindicated drug (such as primaquine) in order to avoid hemolytic complications. [12] Hence, the opportunity was taken to conduct this research to assess the frequency of G6PD deficiency in patients attending Universal College of Medical Sciences-Teaching Hospital (UCMS-TH) and to compare the qualitative and quantitative methods of G6PD estimation.

Method

This study was a hospital based cross sectional and comparative study, conducted in Department of Pathology with collaboration of Department of Biochemistry and Department of pediatrics, UCMS-TH, Bhairahawa, Nepal from March 15 to September 15. Patients attending the hospital with clinical as well as laboratory suspicion like fever and shortness of breath, hyperbilirubinemia, jaundice after antimalarial drug therapy, peripheral smear showing bite cells, Heinz bodies and other hemolytic blood picture were included. Patients of age more than 60 years of age were excluded from the study.

Study population and sample size was determined by using the formula: $n = \frac{Z^2PQ}{D^2}$

n = $(1.96)^2$ ×0.07 × 0.93/ (0.0025 = 100 where, n = sample size, z = critical value = 1.96, P = prevalence of disease = 7%, Q = without disease (1-P), D = allowance error (5%).

The hematological parameters including hemoglobin concentration, Red blood corpuscular (RBC) count, Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and

Mean Corpuscular Hemoglobin Concentration (MCHC) of the patient were obtained from Hematology analyzer 5 parts (Beckman coulter, DxH 520).

Spectrophotometric assay of G6PD

G6PD in RBCs is released by lysing agent present in the reagent. The G6PD released catalyzes the Glucose-6-phosphate with reduction of NADP to NADPH. The rate of reduction of NADP to NADPH is measured as an increased in absorbance at 340 nm produced in the reaction catalyzed by the enzyme which is proportional to the G6PDH activity in the sample. [13] Coral G6PD assay kit (Clinical System, Bambolim complex, Goa, India) was used and the procedure provided in the manual with the kit was followed. The absorbance was taken 2 min after reaction mixture was added with blood and final value was calculated by multiplying absorbance (ΔA) with factor 4778 divided by hemoglobin concentration of the patients. The normal value of the G6PD activity at 37 °C is 6.4– 18.7 IU/g Hb.

Methemoglobin reduction test

The action of nitrate on red cells results in formation if oxidized form, methemoglobin, and in the presence of methylene blue, methemoglobin is reduced to hemoglobin through the oxidative pathway. The absence of glucose 6 phosphate dehydrogenase is determined by unchanged brown colored methemoglobin after addition of methylene blue and incubation. [8] 2.0 ml of blood was taken in in the test tubes marked with ‘positive control’, ‘negative control’ and ‘test’. 0.1 ml of sodium-nitrate-glucose solution and 0.1 ml of methylene blue solution was added in test tubes marked ‘positive control’ and ‘test’ and mixed. No reagent was added in negative control tube. After 3 hours of incubation, 0.1 ml of solutions from each tubes were transferred to clean and labelled new test tubes. The volume was made up to 10 ml and observed for the comparison of ‘test’ with positive and negative controls. If the ‘test’ was clear red similar to negative control, it was interpreted as negative and if it was brown similar to positive control, it was interpreted as positive for G6PD deficiency.

Statistical analysis was done using SPSS IBM ver. 22, Newyork.. The parametric data were expressed in mean ± SD. Categorical data were expressed as frequencies with corresponding percentages and evaluated using Chi-Square test with level of significance set to 0.05 for all tests and P-value < 0.05 was considered significant.

Results

Table 1: Comparison of two Methods (Methemoglobin Reduction Test and Spectrophotometry) of G6PD Analysis

Results	Methemoglobin reduction test	Spectrophotometric assay
Positive	3(3%)	3(3%)
Negative	97(97%)	90(90%)
Hyperactive	Not Applicable	7(7.0%)
Total	100(100.0%)	100(100.0%)

Among 100 subjects under study, both the test methods showed the frequency of G6PD deficiency to be 3%. Methemoglobin reduction test is unable to differentiate subjects with increased enzyme activity. In case of spectrophotometric assay, 90 subjects had a normal enzyme activity and 7 were having increased enzyme activity.

Table 2: Mean Value and Standard Deviation of Different Variables

Hematological Parameters	Sex	Mean ± SD
Hemoglobin (g/dl)	M	9.06 ± 2.37
	F	8.13 ± 2.37
	Total	8.57 ± 2.40
RBC	M	3.28 ± 0.97
	F	3.006 ± 1.13
	Total	3.14 ± 1.06
PCV	M	27.51 ± 7.64
	F	23.34 ± 7.69
	Total	25.65 ± 7.83
MCV	M	85.01 ± 15.62
	F	85.32 ± 16.83
	Total	85.17 ± 16.18
MCH	M	28.15 ± 5.89
	F	27.43 ± 6.08
	Total	27.78 ± 5.97
MCHC	M	32.40 ± 3.31
	F	31.97 ± 1.45
	Total	32.18 ± 2.51
Reticulocyte count	M	1.56 ± 0.87
	F	1.69 ± 0.93
	Total	1.62 ± 0.90
Kinetic Assay Result	M	15.34 ± 4.7
	F	16.01 ± 3.74
	Total	15.69 ± 4.23

Table 2 shows the mean value and standard deviation of different variables under study with differentiation between two sexes.

Table 3: Correlation between Different Variables

Variables		HB	PCV	MCV	MCH	MCHC	Reticulocyte count
Age	r	0.052	0.064	-0.212	-0.242	-0.022	-0.241
	p	0.609	0.524	0.034*	0.015*	0.831	0.016*
RBC	r	0.786	0.821	-0.524	-0.515	-0.270	-0.081
	p	0.001**	0.001**	0.001**	0.001**	0.007**	0.422
G6PD activity	r	-0.100	-0.098	-0.042	-0.118	-0.220	0.289
	p	0.321	0.332	0.676	0.241	0.028*	0.004**

r = Pearson correlation

p = Significance (two-tailed)

* = (correlation is significant at 0.05 level, two tailed).

** = (correlation is significant at 0.01 level, two tailed).

Table 3 shows the correlation between different variables with their Pearson's correlation and level of significance. Age is not statistically significant with MCV and MCH with r value − 0.212 (p-value: 0.034) and − 0.242 (p-value: 0.015) respectively. The correlation between age and reticulocyte count was significant with p-value 0.016 and r value − 0.241.

RBC count is correlated with Hemoglobin, PCV, MCV, MCH and MCHC with r value 0.786, 0.821, -0.524, -0.515 and − 0.270 and p- value < 0.001, < 0.001, < 0.001, < 0.001 and 0.007 respectively. The G6PD activity showed p-value: 0.028 and r value − 0.220 with MCHC while with reticulocyte count they were 0.004 and 0.289 respectively.

Discussion

This study attempted to check the reliability of qualitative method of testing G6PD (Methemoglobin reduction test) in reference to the spectrophotometric method and also to find out the frequency of deficient cases among the patient attending UCMS-TH, Bhairahawa, Nepal.

100 subjects were studied in our research and the frequency of G6PD deficient subjects was found to be 3%. In a study by Oni G *et al*. 60% were male subjects and 40% were female subjects with frequency of G6PD deficient males 40 and that of females 35 in contrast to our study in which, among 52% females and 48% males and all 3 of the deficient individuals were males. [14]

The positive (deficient) results given by both Methemoglobin Reduction Test and Spectrophotometric methods were same that is 3%. The spectrophotometric assay detected 7 individuals to be hyperactive

which could only be considered as normal with methemoglobin reduction test. [15] Since our main concern was with deficiency of the enzyme, methemoglobin reduction test was considered as a good method for screening G6PD deficiency.

Suvitha Thilakarajan *et al*. (2014) stated, in their study that, methemoglobin reduction test was able to pick heterozygous female. Their study tested subjects only with methemoglobin reduction test and suggested to analyze its efficacy with a simultaneous enzymatic assay. On contrast to their study, the present study has adopted both the methods for testing G6PD assay. But in our study, none of the female subjects were found to be G6PD deficient hence we couldn't predict if Methemoglobin reduction could analyze heterozygous females or not. [16]

The frequency of positive subjects in our study was similar to that found in Dhanusa district in previous study by Lamichanne N *et al*. (2017) where it was 3.1%. While it was different than prevalence in other districts like Jhapa where it was 9.8%, in Morang district where it was 5.8%. Our study was hospital based unlike their study. The samples in our study were suspected cases of G6PD deficient patients attending UCMS-TH only which could be low as compared to the susceptible patients of G6PD deficiency regions. [17]

The mean ± SD of G6PD activity in the sample population was 15.69 ± 4.23 U/gHb in our study which is quite different than that of Kim S *et al*. (2011) who projected 10.9 ± 4.6 U/g Hb. Since the study done by them was in different region of Asia i.e. in Cambodia which may vary G6PD enzyme activity geographically. Moreover, their method of assay was different from our study. They performed the tests using CareStart™, a rapid diagnostic test method for G6PD screening while ours was according to G-Six Kinetic Assay, Tulip Group, India, by Spectrophotometric assay. [18]

The male subjects having G6PD deficiency outnumbered the number female subjects in our study, similar to the study by Das K P *et al*. (2013) in which all the deficient subjects were males and the study conducted by Gautam N *et al*. (2019) in which 81.8% of the deficient subjects were males. [19, 20]This suggests the fact that G6PD deficiency is an x-linked genetic disorder and males are more prone to be affected than females.

In our study, the frequency of subjects having increased enzyme activity was 7% which is very similar to the result obtained by Khim *et al*. (2013) in which they have 5.1% of subjects considered in Class V which WHO classifies as a class of individuals having an increased activity of G6PD enzyme. [21, 22] According to Domingo G J *et al*. high count of young red cells or high leukocyte count might result in high G6PD activity because G6PD level is higher in these cells which supports our study in a way that all the subjects with hyperactivity of enzyme were having an increased level of reticulocyte count. [23]

The reticulocyte count was higher in the G6PD deficient subjects similar to result of Al-Nood AH *et al*. (2011), but in our study, the reticulocyte count was higher in all the subjects with hyperactive enzyme status. The mean ± SD of reticulocytes was 1.62 ± 0.90 and P-value was 0.004. [24] In our study 25% of the subjects were having high reticulocyte count and remaining 75% had a normal count.

The G6PD activity was statistically correlated with RBC count with P-value of 0.048 in our study while with PCV there was no significant correlation found. Since the G6PD deficiency precipitates the hemolysis with other factors that might have caused the RBC count to be significantly reduced. Also we didn't find any statistical association between hemoglobin level and G6PD.

Conclusion

Not very frequent but there is presence of G6PD deficiency in patients attending Universal College of Medical Sciences-Teaching Hospital (UCMS-TH). The methemoglobin reduction test is convenient and cheaper. Although it is a time consuming method, for the mass screening it could be very easy because several samples can be tested at once. The positive results given by this method were very satisfying with Spectrophotometric assay in our study even though it was not able to generate exact enzyme activity in the sample. It is advisable to screen suspected patients with methemoglobin reduction test before initiating oxidative drug therapy.

Limitations

In this study, we tried to point out the efficacy of methemoglobin reduction assay with reference to spectrophotometric assay but the study would have been more effective if we had found partially deficient females. We could generate the actual efficacy of methemoglobin reduction test. Also, our study was a hospital based study, exact burden of G6PD deficiency can be assessed by community based study with a larger sample size.

Abbreviations

G6PD: Glucose 6 phosphate dehydrogenase; Hb: Hemoglobin; UCMS-TH: Universal College of Medical Sciences-Teaching Hospital; RBC: Red blood corpuscular; PCV: Packed Cell Volume; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration.

Declarations

Ethical approval and consent to participate: Ethical approval for the research was taken from Institutional Review Committee (IRC), UCMS, Bhairahawa, Nepal (UCMS/IRC/034/19). Both verbal and written consent were taken from the participants and concerned guardians prior the study.

Consent for publication: Not applicable

Availability of data and materials: The data set that was used and analyzed in this study is available from the corresponding author upon reasonable request.

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Author's contribution: Uday Sharma, Satyendra Mishra and Narayan Gautam designed the study in combination. Uday Sharma, Satyendra Mishra and Badri Kumar Gupta carried out the data collection. Uday Sharma, Satyendra Mishra and Narayan Gautam carried out the laboratory analysis. All authors contributed in preparation and approval of the final manuscript.

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