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Assay of Glucose 6-phosphate Dehydrogenase Enzyme and its Correlation with Disease Prevalence in Patients with *Plasmodium falciparum* Malaria

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ABSTRACT

Falciparum malaria is a major global health problem and is third leading cause of death after HIV and tuberculosis. Although, several targets and related drugs are available, yet the parasite evolves a resistance mechanism to most of the existing drugs. Under such circumstances, it is imperative to explore antimalarial drug targets and effective drugs. Malaria is an interesting case of evolutionary selection and several host genetic factors have been selected in response to *Plasmodium falciparum* infection, such as hemoglobin variants, glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase deficiency. Among these G6PD deficiency is one of the most studied host genetic factor that confer resistance to malaria in endemic and non endemic region. We have studied G6PD deficiency in *Falciparum malaria* patients and ethnically matched controls in non endemic region of 170 blood samples. G6PD deficiency in samples was detected by Fluorescent spot test. The samples were incubated with reaction mixture, spotted and visualized (366 nm) under ultraviolet light. Results indicate that the frequency of % G6PD deficiency is 9.2 in males and 7.14 in females in non endemic region. On the basis of our data and earlier studies, we conclude that G6PD deficiency is more prevalent in those areas where the frequency of malaria infection is high. G6PD is a selective force against the pressure of malaria in endemic regions while in non endemic regions the burden of malaria is seasonal and low which accounts for the deficiency of enzyme in conferring protection against malaria.

Key words: *Falciparum malaria*, host genetic factor, glucose-6-phosphate deficiency, non endemic region

INTRODUCTION

Malaria is a leading cause of death after HIV and tuberculosis affecting 500 million people per year (WHO, 2010). Although, ubiquitous in occurrence, African sub-continent is strongly affected (Miller, 1994). Several endemic regions have been reported in India such as Rourkela, Chhattisgarh and Madhya Pradesh. Study of host genetic factor reveals that several candidate genes have been known to play an important role in malaria prevalence in endemic as well as non endemic regions. Glucose-6-phosphate dehydrogenase (G6PD) is an important housekeeping enzyme in the pentose phosphate pathway. The balance of reduced nicotinamide adenine

dinucleotide phosphate (NADPH) (a necessary cofactor for cell detoxification) is maintained by G6PD. Moreover, G6PD is the sole generator of NADPH in the red blood cells and may alone prevent oxidative damage and severe anemia. G6PD deficiency is the most commonly known enzymopathy that plays a role in malaria. A wide variety of G6PD variants with reduced enzyme activity have been reported (Luzzatto *et al*., 2001). Such mutations in G6PD genes responsible for reduced enzyme activity played role in resistance to malaria and are considered as the best examples of selection in the human genome (Verrelli *et al*., 2002).

G6PD deficiency has been implicated in clinical disorders, such as neonatal jaundice, hemolytic anemia and several cardiovascular diseases (Beutler, 1994). Inspite of its role in many clinical disorders, it has been suggested that G6PD deficiencies are selectively maintained in *Falciparum malaria*. G6PD deficiency is strongly associated with the distribution of malarial endemicity and many variants have been found at low-to-high frequencies in different populations (Vulliamy *et al*., 1992). Classic G6PD A/B polymorphism results from a single amino acid replacement. The B variant, with normal enzyme activity, dominates in frequency worldwide and have been found as ancestral state by comparison with chimpanzee G6PD (Kay *et al*., 1992). G6PD with A variant in exon 5, possesses 85% enzyme activity and is found in sub-Saharan Africa at frequencies as high as 40% but rarely reaches frequencies 11% outside Africa and the Middle East (Beutler, 1994; Ruwende *et al*., 2002). G6PD deficiency has also been studied in various populations in endemic region of India and Iran (Balgir, 2006; Iranpour *et al*., 2008). In the present study, we have studied G6PD enzyme deficiency assay in *P. falciparum* malaria patients and control blood samples in non endemic region of Uttar Pradesh and compare the finding with the published data in the endemic region (Orissa).

MATERIALS AND METHODS

Sample collection: Blood samples were taken from, Maha Maya Govt. Medical College, Akbarpur, Ambedkernagar and various clinics in adjacent areas. In the sample collection ethical guideline was followed. The blood was drawn from each individual and collected in citrate buffer (3.8% sodium citrate) to prevent coagulation. An aliquot of 20 μL was used for glucose 6-phosphate dehydrogenase assay. A total 170 blood samples (Controls and patients) were included in this study.

Glucose 6-phosphate dehydrogenase (G6PD) assay: G6PD assay in RBCs was assayed using the semi-quantitative fluorescent spot test. In this method, Glucose-6-phosphate (G6P) is oxidized into 6-phosphogluconate by G6PD present in blood using NADP as a cofactor. NADP is reduced to NADPH which fluoresces under long UV light. However, to continue the reaction for a longer time with limited NADP, oxidized glutathione (GSSG) is added to the reaction mixture. GSSG oxidises NADPH and itself gets reduced (GSH) through the action of Glutathione Reductase (GR) present in blood. For the assay, 10 μL of RBCs were subjected to lyses with 90 μL of sterile distilled water. About 10 μL of lysed RBCs were added to 100 μL of the reaction buffer (0.1 M glucose 6-phospahte, 0.75 M Tris-Cl pH 8.0, 1% saponin, 0.007 M NADP, 0.008 M GSSG), the contents were mixed thoroughly and a zero time spot (1 cm diameter) of the blood-reagent mixture was applied on 3 mm Whatman filter paper. This spot served as the background control. The reaction was incubated at 37°C and samples were spotted after incubation for 10, 30 and 60 min, respectively next to the control spot on the filter paper.

Visualization of spots: The spots of samples were allowed to dry at room temperature and examined under long wave (366 nm) UV light for fluorescence. The absence of fluorescence uptil

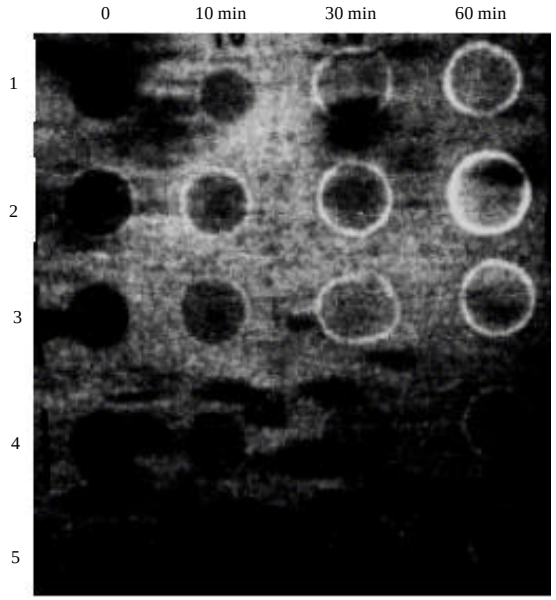

Fig. 1: G6PD assay. Spots at different time points after start of the reaction (0, 10, 30 and 60 min) as observed under UV light. Samples 1, 2 and 3 fluoresce maximally (high G6PD activity) while sample 4 fluoresced weakly starting at 30 min (weak G6PD activity) and sample 5 did not fluoresce at any time point (G6PD deficient)

30 min indicated the deficiency of G6PD in the individual (Fig. 1). The intensity of fluorescence at 10 and 30 min was also an indicator of the level of G6PD in individual RBCs.

RESULTS AND DISCUSSION

We have screened the deficient and non deficient blood samples on the basis of the following observations. The spot of samples at several time intervals are shown in Fig. 1.

Normal (non-deficient) samples:

Zero min. spot:	No fluorescence
10 and 30 min. spot:	Strong fluorescence

Deficient samples:

Zero min. spot:	No fluorescence
10 and 30 min spot:	No fluorescence
60 min:	Weak/no fluorescence

In case of deficient samples, very weak or no fluorescence were observed after prolonged incubation even after 90 min. In our study in non endemic region of India (Uttar Pradesh), out of 120 males 13 (9.23%) and of 50 females, 7 (7.14%) were found to be G6PD deficient. While an earlier study by Balgir (2006) pointed that the G6PD deficiency in endemic region was reported to range from 10.7-17.4%. Highest G6PD deficiency in endemic region was reported in Praja population.

G6PD is an important housekeeping enzyme which catalyzes the first step of the pentose phosphate pathway which is the sole source for the production of reducing capacity in the form of NADPH in erythrocytes. NADPH generated by this pathway is used to reduce glutathione which is used by the cells to neutralize free radicals produced during oxidative stress. G6PD deficiency is the most common enzymopathy reported worldwide. A number of genetic variants have been discovered in many populations that may cause G6PD deficiency or reduced enzyme activity (Luzzatto *et al*., 2001). Tishkoff *et al*. (2001) determined haplotypes of two polymorphisms associated with low activity allele (A^-) of G6PD which is responsible for decrease in G6PD activity. These polymorphisms have evolved independently within the past 3000-10,000 years. Similarly, the trait cause sickle cell disease and α-thalassemia also has origins within this time frame (Flint *et al*., 1993; Currat *et al*., 2002). Although, G6PD deficiency results in a number of clinical disorders like neonatal jaundice, hemolytic anemia and cardiovascular disorders (Beutler, 1994), it is selectively maintained in some populations across the globe. Erythrocytes deficient of G6PD cannot produce NADPH and reduced glutathione, thus impairing the cell's ability to combat oxidative damage caused by free radicals, ultimately resulting in hemolysis. *P. falciparum* parasite, during its erythrocytic cycle, produces free radicals as a result of metabolism which may cause hemolysis in the absence of G6PD and hence the death of parasite. It may be due to this reason that the G6PD deficiency is maintained as a balanced polymorphism in malaria hyperendemic regions worldwide. The infection of parasite in human host alters several biochemical parameters. In mice there is significant increase in plasma total protein, globulin, erythrocyte fragility, total bilirubin, oxidative stress, glucose-6-phosphate dehydrogenase (G6PD), liver superoxide dismutase (SOD) and catalase (CAT) enzyme activities (Iyawe and Onigbinde, 2009). Severe malaria and malaria-typhoid co-infection cause significant alteration in hematological and biochemical parameters (Kayode *et al*., 2011).

G6PD deficiency information on various populations of India also shows distinct patterns of distribution (Tishkoff and Williams, 2002; Sukumar *et al*., 2004; Balgir, 2006) (Fig. 2a, b). A number of known and novel polymorphisms have been reported in Indian populations. Among them, the A^- mutation is almost absent from the Indian populations (Tishkoff and Williams, 2002). A novel SNP, the G6PD-Orissa (Kaeda *et al*., 1995) was discovered in some tribal populations of Orissa where malaria is hyper-endemic. Strikingly, the Med mutation which is present in considerable frequency in other parts of India, was found to be completely absent in Orissa. Taken

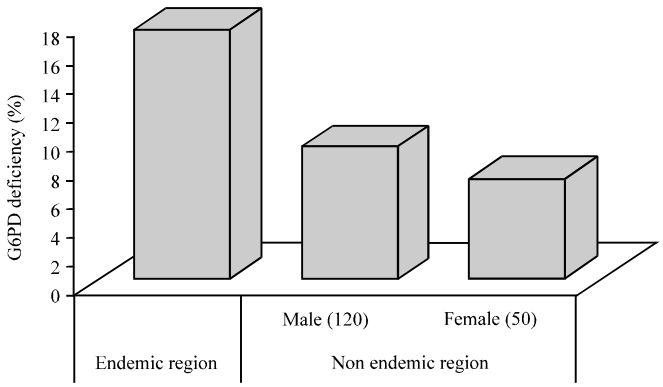

Fig. 2a: Graphical representation of G6PD deficiency in *P. falciparum* malaria patients and controls in non endemic and endemic region of India

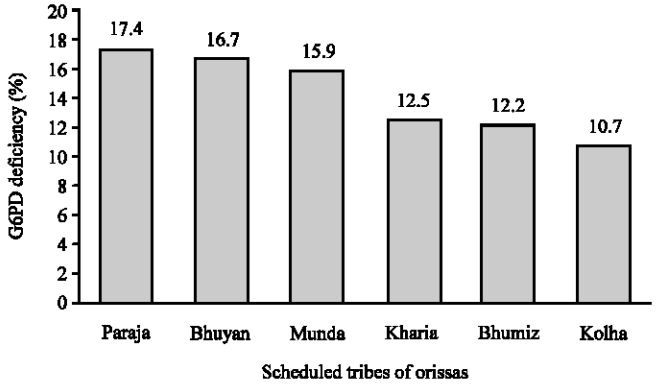

Fig. 2b: The distribution of G6PD deficiency among major scheduled tribes of Orissa (Data were taken from published paper of Balgir (2006)

together, the G6PD deficiency data on India shows that the deficiency trait is favored as the function of malarial prevalence within the population (Balgir, 2006) but the exact molecular basis of this is still unclear. More exhaustive studies on the spread and distribution of individual mutations and linkage disequilibrium (LD) patterns across the G6PD locus may explain the molecular evolution of G6PD deficiency trait driven by disease pressure. In India the frequency of sickle cell allele decreases in a malaria endemic tribal population and the frequency of G6PD enzyme deficiency and beta-thalassemia allele increases and vice versa. Sickle cell haemoglobin (Hb S, $\beta^{6\,GAG\rightarrow GTG}$) in North East part of India is restricted to the tea garden labour communities and a positive correlation ($R^2 = 0.703$) of β^E-globin gene frequency and mean incidence of *P. falciparum* infection in malaria endemic zones have been reported (Sharma and Mahanta, 2009). The other genetic polymorphism including thalassemia and G6PD deficiency may also probably be associated with the distribution of HbE in malaria endemic zones of Northeast India (Sharma and Mahanta, 2009). Sickle cell, beta-thalassemia and G6PD mutation alleles have probably evolved as a protective mechanism against *falciparum* malaria by natural selection. On comparing our data with published data in endemic regions (regions, where disease frequency is higher throughout the year), we observed that the percentage deficiency was higher in the endemic region as compared in malaria non-endemic region (Fig. 2a, b).

In a comparative study of malaria infections in endemic areas of Asia and Africa the distribution of G6PD deficient allele is strongly associated with malaria endemicity (Osamor, 2010). The cells with G6PD deficiency lack the ability to resist sustained oxidative stress adequately and hence the free radical producing parasite is a challenge to such cells. This situation is thought to make them more susceptible to phagocytosis (Jeremiah *et al*., 2008). Malaria infection has been found to be associated with lipid peroxidation accompanying reduction in antioxidant capacity of the infected patients. Malondialdehyde (MDA) (a biomarker of lipid peroxidation) was evaluated in adult Nigerian patients with *P. falciparum* and *P. vivax* malaria infection (Idonije *et al*., 2011).

The gene for G6PD enzyme is located on the telomeric region of long arm of the X chromosome (at Xq28) (Nathans *et al*., 1986). In several studies, the male to female ratio in G6PD deficient was detected. The male to female ratio in G6PD deficient neonates was reported in Iran (Iranpour *et al*., 2008), the city of Dhahran (Saudia Arabia) (Mallouh *et al*., 1992), Yanbu (Saudi Arabia) (Muzaffer, 2005), Punjab (India)(Verma *et al*., 1990), Tehran (Iran) (Abolghasemi *et al*., 2004) as 5.5:1, 2:1, 3:1, 1.8:1 and 6:1, respectively. It seems that the incidence

of G6PD deficiency in the Iranian female population is lower than in other female populations. The existence of gender difference in enzyme activity and a significantly higher G6PD deficiency in males have been reported in Iran (Iranpour *et al*., 2008).

In our study on *P. falciparum* malaria in non endemic adjoining areas of Uttar Pradesh (Faizabad). Samples of malaria patients and controls were screened and it was found that the frequency of % G6PD deficiency is 9.2 in males and 7.14 in females in malaria non endemic region. Glucose-6-phosphate deficiency is more frequent in those areas where the frequency of malaria infection is high. As compared to endemic region like Orissa and Jharkhand (where, frequency of malaria is high throughout the year), in the adjoining areas in Faizabad and Ambedkarnagar, there is low and seasonal infection of malaria. In an earlier study in endemic region the G6PD deficiency in several populations were identified and all studied populations have high frequency of G6PD deficiency. Praja population has highest frequency of G6PD. Comparison of results indicates that high frequency of G6PD enzyme deficiency has been selected as the part of protective effect. Our results indicate that geographical distribution of G6PD deficiency is consistent with evolutionary selection by malaria and in endemic region (like Orissa) glucose 6 phosphate deficiencies are strongly associated with protection against severe malaria. So it can be concluded that in nonendemic region glucose 6 phosphate deficiencies is associated with protection against malaria.

CONCLUSION

Due to multidrug resistance in *P. falciparum* malaria parasite, there is an urgent need of develop new therapeutics to combat malaria. The host genetic factor seems to play an important role in development and prognosis of disease. Under such circumstances the host genetic factors must be explored. Malaria is an example of evolutionary and balancing selection implying that certain traits in human host are selected in response to disease pressure to provide resistance. Glucose-6-phosphate dehydrogenase deficiency is one of them. In endemic region (where, frequency of disease is high through out year) G6PD deficiency is selected against the pressure of malaria. In non endemic region, low frequency of G6PD deficiency is consistent with low or seasonal transmission of malaria.

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