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MALARIA PROTECTION IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENT INDIVIDUALS IN BAMENDA POPULATION OF CAMEROON

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ABSTRACT

The high frequency of glucose-6-phosphate dehydrogenase (*G6PD*) deficiency gene in malaria endemic regions is believed to be due to the enzyme deficiency advantage against fatal malaria. However, the mechanism of this protection is not well understood and therefore was investigated by comparing differences in plasmodial parasitaemia, full blood count profile and the severity of clinical malarial symptoms of *G6PD* deficient and *G6PD* non–deficient cohort groups in the population. Our results showed that 10.4% (63/606) of those tested carried the *G6PD* deficiency gene. *G6PD* deficient heterozygous females and hemizygous males manifested significantly reduced ($P<0.05$) parasitaemia, less severe malarial anaemia, less severe clinical malarial symptoms and elevated haematological profile during the progress of malaria when compared with *G6PD* non–deficient subjects. The results seem to suggest that *G6PD* deficiency gene could be among the factors that confer malaria protection in the hosts through reduction of severe malarial anaemia, density parasitaemia and clinical malarial symptoms while having less effect on frequency of infection *perse.*

KEYWORDS: *G6PD* deficiency, malaria, haematological profile

INTRODUCTION

Malaria is caused by the mosquito–borne haematoprotozoan parasite of the genus *Plasmodium* and transmitted by the female anopheles mosquito. It is a severe public health problem in tropical areas and a vast majority of mortality and morbidity is caused by infection with *P. falciparum.* At least 90% of the deaths occur in Sub-Saharan Africa (Tsuji *et al.,* 2001; Greenwood & Mutabingwa*,* 2002). Between 300 – 500 million clinical cases are reported each year and an estimated yearly mortality rate of over one million deaths (Felger *et al.,* 2003).

Glucose-6-phosphate dehydrogenase (*G6PD*) deficiency is one of the most common inherited single gene disorders in the world (Kar *et al.,* 1990). *G6PD*, a key enzyme in the pentose phosphate pathway (PPP), catalyses the production of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) within the erythrocytes. This reduced cofactor is used to regenerate reduced glutathione (GSH), to maintain a reduced environment within the red cell, to enhance the ability of the cell to withstand haemolysis that could result from oxidative damage, characteristic of or following exposure to oxidant drugs (Lui *et al.,* 1994, Cheesbrough, 2000). The protection against malaria severity conferred by *G6PD* deficiency in malaria endemic regions of the world has been widely speculated. However, the mechanism by which the *G6PD* deficiency gene conveys resistance to malaria is not properly understood. Resistance to malaria by *G6PD* deficient individuals is thought to be due to increased oxidative stress in *G6PD* deficient cells, hence, red cell/parasite damage (Lui *et al.,* 1994). Although protection against malarial mortality has been indirectly estimated using gene frequencies, the degree of protection has not been determined using cohort studies. In this study, therefore, the current incidence of *G6PD* deficiency and its protective effect on malaria, were investigated in Bamenda population of Cameroon with intent to throw more light into the role this factor could play in the protection against malaria.

MATERIALS AND METHODS

Study area: Cameroon, a malaria endemic central African country has high malaria infection rates all-year-round throughout the country. Bamenda is in the North Western part of the country and incorporates about 2% of Cameroon's total population. The populace has very low knowledge of *G6PD* deficiency.

Subjects and blood sample collection: A total of 606 volunteers aged between 3 to 70 years, who sought for malaria treatment at the Bamenda Provincial Hospital and other volunteers in the community participated in the study. Blood samples (5ml) were collected by venipuncture into sample tubes containing EDTA as anticoagulant and then rocked gently to mix the blood and the anti-coagulant. However, blood samples (0.1ml) for routine monthly malaria test were collected by thumb pricking using lancets. Volunteer cohort groups, comprising forty (40) *G6PD* deficient and 40 *G6PD* non-deficient dominant homozygotes (HbAA) were selected from those tested and monthly monitored for the frequency of plasmodial infection, haemoglobin concentration, parasite density, percentage parasitaemia, blood sugar level, and other clinical malaria presentations such as respiratory distress, high fever, severe headache, diarrhoea, vomiting, weakness and anaemia for a period of one year, whenever they show signs of malaria infection. Selection was based on the individual's accessibility and willingness to take part in monitoring for the plasmodial infection.

Malaria diagnosis: Malaria was diagnosed based on clinical presentations of malaria symptoms and confirmed by microscopic examination of Giemsa or Leishman stained thick and thin blood films for malaria parasite.

Parasitaemia and parasite density determinations: Parasitaemia was microscopically determined from the total number of infected and uninfected RBCs counted in 1000 RBCs in a thin film (oil immersion, X100), using a hand tally counter according to the method of Greenwood & Armstrong, (1991). Percentage parasitaemia was then computed thus:

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$$\% \text{ Parasitaemia} = \frac{\text{Total number of infected RBCs}}{\text{Total number of RBCs (infected and uninfected)}} \times 100$$

Parasite density was estimated by counting the number of intra-erythrocytic parasites in 10,000 RBCs (approximately 36-45 oil immersion microscopic fields) (Greenwood & Armstrong, 1991). The parasite density per μl was then calculated as follows:

$$\text{Parasite density}(/\mu l) = \frac{\text{RBC count } (/\mu l) \times \text{Number of parasites per 10,000 RBCs}}{10{,}000}$$

Glucose oxidase reagent for fasting blood sugar (phenol free): The glucose reagent (Teco Diagnostic) was reconstituted by mixing 50ml of distilled water with each bottle of reagent. The reconstituted reagent contained 15μl/ml glucose oxidase, 1.2μl/ml Peroxidase (horseradish), 4.0 μl/ml mutarotase, 0.38mM 4–minoantipyrine, 10mM p–hyroxybenzene sulfonate and non-reactive ingredients. The reagent was stored at 2 – 8^{o}C in amber containers.

Clinical symptoms in the cohort group during malaria infection: The cohort groups were observed for clinical symptoms during malaria infection by the medical doctors in the hospital in the area of study. The intensity and severity of the symptoms were rated from 1 to 5 (+ to +++++) in the increasing order of severity. 0 means that the symptom was not observed.

Determination of G6PD deficiency: The activity of G6PD was qualitatively determined by fluorescent spot test as described by Beutler *et al.* (1996). The reaction mixture contained glucose-6-phosplate (0.01M), $NADP^{+}$ (0.01M), saponins (0.02M), phosphate buffer, pH 7.4 and distilled water. Fluorescence was produced due to the reduction of $NADP^{+}$ to NADPH. This reaction is coupled to oxidation of glucose-6-phosphate to 6-phosphogluconolactone and catalysed by G6PD. Specimen with G6PD activity of <20% of normal (severe deficiency) do not fluoresce as the small amount of NADPH formed is reoxidised by glutathione present in the reagent. Presence of fluorescence indicated normal cells while weak fluorescence indicated slight deficiency.

Full blood count: Haematological indices [erythrocyte counts, white blood cell count (WBC), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), packed cell volume (PCV) or haematocrit (HCT), Hb concentration and platelet count (PLT)] were analysed using an automated system, CELL-DYN 1200 Hematology Analyser (Abbott, USA).

Determination of serum fasting blood sugar: Glucose (1.5 ml), reconstituted reagent was pipetted into clean cuvettes labeled blank, standard and tests and incubated for 5 minutes at 37^{o}C. Then 10μl of standard and test sera were added to the respective cuvettes and incubated for another 10 minutes. The amount of coloured complex formed was proportional to glucose concentration and can be photometrically measured at the wavelength of 520nm against the reagent blank. The glucose concentration in the subject's serum was then calculated using the formula:

$$\text{Fasting Blood Sugar} = \frac{\text{Absorbance of Standard}}{\text{Absorbance of Test}} \times \text{Conc. of Standard (mg/dl)}$$

Haemoglobin (Hb) genotype: Haemoglobin genotypes were determined by cellulose acetate membrane electrophoresis (CAME) of Evans (1971) as modified by Uzoegwu (2001).

RESULTS

Prevalence of *G6PD* deficiency in Bamenda population: The result of the *G6PD* status in the Bamenda population (*Table 1a & 1b*) suggest that the prevalence of the enzyme deficiency in males (9.2%) is significantly higher (P<0.05) than that in females (1.2%) resulting in a 10.4% prevalence of the enzyme deficiency in the population.

Table 1a: *G6PD* Activity

Observation	No of Subjects
Fluorescence (Normal Activity)	89.6% (543/606)
Weak Fluorescence (Low Activity)	4.45% (27/606)
No Fluorescence (Severe Deficiency)	5.95% (36/606)

Table 1b: *G6PD* deficiency in male and female population in Bamenda

Sex	*G6PD* deficiency prevalence (%)
Male	9.2 (56/606)
Females	1.2 (7/606)
Total	10.40 (63/606)

Clinical symptoms during malaria infection in the cohort groups: The results of clinical symptoms during malaria infection (*Table 2a*) reveal that a greater number of *G6PD* non–deficient subjects suffered more frequent and more severe malaria attack while *G6PD* deficient subjects had significantly fewer episodes of severe malarial anaemia, few episodes of high parasitaemia and less severe clinical symptoms. Although the average fasting blood sugar of *G6PD* non-deficient subjects was lower than that of *G6PD* deficient subjects, the difference was not statistically significant (P>0.05) (*Table 2b*).

Table 2a: Protective effect of sickle and *G6PD* deficiency genes against malaria morbidity

Clinical malarial symptoms	*G6PD* non-deficient subjects (n = 40)	*G6PD* deficient (n = 40)
	N^{o} of cases observed in the cohort groups	
Severe malarial anaemia episodes (Hb<6g/dl and >10,000 Parasitaemia)	5	-
All cause severe anaemia episodes (Hb<6g/dl and any level parasitaemia)	6	2
High density parasitaemia episodes (>10,000 Parasitaemia)	5	-
Cerebral malaria episodes	3	-
Hypoglycaemic episodes	7	3
Coma	1	-

Seizure	2	-
Respiratory distress	2	1
Severe headache	10	5
Diarrhea	8	3
Severe vomiting	6	2
Av. frequency of malaria attack	4	1

Table 2b: Average fasting blood sugar concentration in malaria subjects

	***G6PD* non-deficient (n = 40)**	***G6PD* deficient (n = 40)**
Fasting blood sugar	66.5 ± 13.6	75.3 ± 24.4

Note: Normal value (70 – 105mg/dl)

Annual frequency of malaria attack in different *G6PD* status: As per the data extracted from the questionnaires and hospital files, the frequency of malaria attack in different *G6PD* deficient and non-deficient subjects is presented in table 3. The results seem to indicate that the frequency of malaria attack is more in *G6PD* non-deficient subjects than in *G6PD* deficient ones.

Table 3*:* Annual frequency of malaria attack of *G6PD* deficient and non–deficient individuals

	Annual frequency of malaria attack							
	≤ 2x		**2 – 5x**		**≥ 5x**		**Total**	
	N^{o}	**%**	**N^{o}**	**%**	**N^{o}**	**%**	**N^{o}**	**%**
***G6PD* deficient**	42	66.7	11	17.5	10	15.9	63	100
***G6PD* non-deficient**	133	24.5	245	45.1	165	30.4	542	100

Haematological profile and parasitaemia in plasmodial infected *G6PD* deficient and non-deficient subjects: Data in table 4 shows that *G6PD* deficient subjects suffered less malaria attacks, had significantly (P<0.05) lower parasite densities and percentage parasitaemia but significantly higher (P<0.05) Hb concentration, RBC, PCV, MCV, MCH and platelet count and lower WBC and RDW though not statistically significant (P>0.05) than in non-deficient individuals.

Table 4*:* Haematological profile and parasitaemia in malaria infected subjects with or without *G6PD* deficiency

Haematological variable / Parasitaemia	***G6PD* non-deficient subjects (n = 40)**	***G6PD* deficient subjects (n = 40)**	
Hb (g/dl)	9.6 ± 2.1	11.7 ± 1.04	*
PCV (%)	22.5 ± 4.6	26.9 ± 2.5	*
RBC (M/μl)	3.2 ± 0.7	3.6 ± 0.4	*
MCV (fl)	72.2 ± 8.4	74.5 ± 7.1	*
MCH (pg)	30.9 ± 4.1	32.4 ± 3.2	*
MCHC (g/dl)	42.8 ± 2.3	43.5 ± 1.6	ϕ
Platelets (K/μl)	258.7 ± 119.7	270.8 ± 88.8	*
WBC (K/μL)	7.5 ± 4.9	7.1 ± 3.2	*
RDW (%)	14.8 ± 3.3	13.5 ± 2.4	ϕ
Parasite density (N^{o}/μl)	1291 ± 259.2	775.3 ± 158.7	*
% Parasitaemia	0.4 ± 0.1	0.2 ± 0.08	*
Annual frequency (N^{o} of times)	2 – 4	< 2	*

Note: Data is presented as: Mean ± Standard deviation

* = Significant (P<0.05) ϕ = Not Significant (P>0.05)

Severity of malaria attack in *G6PD*–deficient and non-deficient subjects: Severity increased as the haemoglobin concentration decreased while parasite density increased (*Table 5)*.

Table 5: Hb concentration and parasitaemia in cohort groups during malaria attack

Hb (g/dl)	**Severity**	***G6PD* non-deficient (n = 40)**	***G6PD* deficient (n = 40)**
		N^{o} of cases observed	
< 6	++++	7	1
6 – 8	+++	8	5
8 – 10	++	12	14
10 – 12	+	13	20

Parasite Density (N^{o}/μl)	**Severity**	***G6PD* non-deficient (n = 40)**	***G6PD* deficient (n = 40)**
		N^{o} of cases observed	
> 10000	+++++	3	-
8000 – 10000	++++	9	-
6000 – 8000	+++	10	1
4000 – 6000	++	9	5
2000 – 4000	+	9	1

DISCUSSION

The high frequency of *G6PD* deficiency gene in malaria endemic populations of tropics and sub-tropics could be due to the enzyme deficiency gene advantage against fatal malaria (Aruoma, 1993). The possible protective mechanism of this protection was investigated in the population of Bamenda district of Cameroon which was earlier revealed to have malaria prevalence of 42.2%, most of the plasmodial infection (93.7%) being caused by *Plasmodium falciparum* (Awah & Uzoegwu, 2007). The 10.4% incidence of *G6PD* deficiency gene screened for this population during this study is similar to most of the incidences reported for most of the malaria endemic areas of the world (El-Hazmi & Warsy, 1994; Allison, 2004). Our major observation in this study that *G6PD* non-deficient individuals were more vulnerable to malaria disease and subsequently exhibited more severe malarial anaemia and symptoms, higher parasite density, more frequency of plasmodial attack and lower Hb concentration during plasmodial infection than the enzyme deficient cohorts seem to support the hypothesis that this enzyme deficiency might have arisen in a bid to fight against *falciparum* malaria (Kar *et al.,* 1990). Because severe anaemia could be provoked by malnutrition and iron deficiency usually associated with the people of the developing countries of Africa, only those who were adjudged to be nutritionally balanced and at balanced diets, at least for the period of the study were selected for the study. It is noteworthy that severe drug-induced haemolysis and rhabdomyolysis were not observed in this study, perhaps due to the fact that only quinine sulphate (200mg) and not chloroquine sulphate or other anti-malarial drugs were used as the main anti-malarial drugs in this area of study. Furthermore, the haemolysis observed in *G6PD* deficiency could not have been food-induced since fava beans or any other dietary factors that could induce haemolysis in G6PD deficient individual are not known to be consumed in Bamenda. *G6PD* deficiency defect is known to be harmless unless red cells are challenged by primaquine, dietary factors such as fava beans or parasite infections such as malaria. Plasmodial infection is likely to induce oxidative stress to the erythrocyte membrane (Aruoma, 1993). It is therefore likely that the differences in haematological indices, percentage parasitaemia and parasite densities of the *G6PD* deficient and non–deficient cohorts could have been as a result of plasmodial infection. Decreased parasitaemia as observed in *G6PD* deficient group in this study is consistent with similar observation reported by Allison and Clyde (1961). Based on the aforementioned observations, it is reasonable therefore, to adduce that malaria parasites could grow better in the RBCs of *G6PD* non-deficient individuals than in the RBCs of the *G6PD* deficient ones, which could result to the high parasite density observed in non–deficient subjects. Our result, therefore, favours the malaria / *G6PD* deficiency hypothesis, which affirmed that *G6PD* deficiency, are contributing factors to malarial resistance (Usanga & Luzzatto, 1985). The result is also supported by the observation that more cohort *G6PD* non-deficient subjects, particularly children below 5 years of age were admitted in the hospital for the treatment of severe malaria than cohort *G6PD* deficient subjects during which severe malaria associated with symptoms including cerebral malaria, seizure, severe headache, vomiting, diarrhoea and respiratory distress, were evident. Furthermore, more episodes of severe malarial anaemia (Hb <6g/dl), hypoglycaemia, and high parasitaemia (>10000parasites/μl) were more evident in well nourished *G6PD* non-deficient subjects compared to *G6PD* deficient ones. Our results also revealed lower morbidity and mortality rates caused by *P. falciparum* since both factors are related to Hb concentration and parasite densities in malaria infection (Field, 1949). However, since severe malaria may occur in the face of occult malaria infection when the parasites are sequestered and their growth cycle is tightly synchronized, the use of parasitaemia may be an unreliable guide to malaria severity. The existence of more severe anaemic episodes in children below 5 years old could be due to the more susceptibility of their red blood cells to haemolysis and is consistent with the observation of Snow *et al.* (1997). Hypoglycaemic episodes, most common in children with severe malaria (Molyneux *et al.,* 1989) were revealed to be more common in *G6PD* non-deficient malaria infected children than in *G6PD* deficient ones; possibly due to increased consumption of glucose in the peripheral tissues by the parasites (Dekker *et al.,* 1997). Furthermore, more malaria–derived products and/or quinine could directly or indirectly stimulate the β-cells of the pancreatic Islets, leading to hyperinsulinaemia and consequently hypoglycaemia in adults subjects as observed in this study. From the results of this study, it is likely that *G6PD* deficiency gene inheritance confers some protection against severe malaria attack by reducing parasite density and consequently reducing debilitating symptoms. But the mechanism is not still clear and is presently being investigated. To understand clearly, the protective role of *G6PD* deficiency gene during the progress of malaria, it is suggested that, the mechanism of intravascular haemolysis of both parasitised and unparasitised red blood cells in malarial attack should be elucidated and also the detrimental effects of *G6PD* deficiency gene in the proliferation of *P. falciparum* investigated at the molecular level.

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