PREVALENCE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY IN CHILDREN ENROLLED IN A MALARIA VACCINE CLINICAL TRIAL IN WESTERN KENYA

By

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I56/7434/02

A thesis submitted in partial fulfillment for the degree of Master of Science (Biotechnology) in the School of Pure and Applied Sciences of Kenyatta University

AUGUST 2007

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or any other award.

Signed _____ Date 13.8.07.

Carol Wangui Hunja

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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Dr. John N. Waitumbi (DVM, PhD)

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DEDICATION

This thesis is dedicated to:

- God, in whom I can do all things through Christ.
- My parents Rose Wanjiku and James Hunja Kitenge.

ACKNOWLEDGEMENT

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ABSTRACT

Deficiency of Glucose-6-phosphate dehydrogenase (G6PD) is the most frequently encountered genetic abnormality of red blood cell (RBC) metabolism, and is estimated to affect over 200 million people worldwide. Malaria is believed to have been the selection pressure that has favored the maintenance of this potentially deleterious trait. The abnormality gives rise to hemolysis under conditions of oxidative stress such as those caused by ingestion of certain drugs or foods, exposure to certain chemicals, infection or hypoxia. G6PD deficiency has been shown to be protective against severe malaria and may confound the interpretation of malaria intervention studies. The objective of this study was to investigate the prevalence of the common African forms of G6PD deficiency namely G6PD A and A- among a group of children targeted to receive a blood stage malaria vaccine. Blood samples were collected from individuals presenting for screening and G6PD genotypes were determined by a combination of the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Of the 570 individuals, 338 (59.3 %) had the normal G6PD B genotype, 153 (26.8 %) had the G6PD A genotype and 79 (13.9 %) had a combination of 376 A→G mutation and 202 G→A mutation that defines the deleterious G6PD A- genotype. The gene frequencies for Gd^{B}, Gd^{A} and Gd^{A-} alleles were 0.69, 0.21 and 0.10 respectively. To determine whether the G6PD locus in the studied population was in Hardy-Weinberg equilibrium, the observed and expected genotypic frequencies in the females were calculated from the Hardy-Weinberg equation and chi-square test used to determine whether there was a significant difference between the observed and expected genotypic frequencies. A statistically significant difference was found between the observed and expected ($p = 0.05$) leading to the conclusion that the inheritance of G6PD deficiency is not in Hardy-Weinberg equilibrium. This implies continuous selection pressure on the Gd^{A-} allele. Data emanating from this study will be used in the interpretation of malaria vaccine efficacy when the study is eventually un-blinded.

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TNT – Trinitrotoluene

Tris-HCl – Tris (hydroxymethyl)-aminomethane hydrochloric acid

WHO – World Health Organization

CHAPTER ONE

1. INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND OF THE STUDY

Malaria is a major public health problem in tropical areas, and is estimated to cause 1 to 3 million deaths and 300-500 million infections annually (Desowitz, 1991). The vast majority of morbidity and mortality from malaria is caused by *Plasmodium falciparum.*

There is strong evidence that G6PD deficiency, an X-linked disorder that affects approximately 400 million individuals in tropical countries is protective against clinical malaria. The mechanisms for protection are not well understood but are thought to include reduced parasite invasion, inhibition of parasite growth due to impaired compensation of oxidative stress, increased lysis and erythrophagocytosis of infected cells (Ruwende and Hill, 1998; Destro-Bisol, 1999; Mehta *et al.*, 2000).

Worldwide, more than 300 genetic variants of G6PD have been described and are categorized according to the degree of enzyme deficiency associated with the mutations. This can vary from normal enzyme activity, moderate deficiency to severe deficiency that is associated with hemolytic anemia. G6PD B is the wild genotype. G6PD A, a common variant in the African population demonstrates normal enzyme activity and is not associated with hemolysis. G6PD A- has an unstable enzyme that has a shortened duration of activity resulting in decreased enzyme activity and is present in about 10-15% of African males and about 2% of females of the same racial background. It is estimated

to be the most predominant variant in Sub-Saharan Africa (Hirono, 1988; Beutler *et al.*, 1989).

Western Kenya is a malaria holoendemic region and very little information on prevalence of G6PD deficiency is available. A study carried out by Moorman *et al.* (2003), in Western Kenya, showed a G6PD deficiency prevalence of 7%. Various malaria vaccine interventional studies are continuously executed in this area (Jose et al., 2006 a, b), and the study reported here represents the first attempt to integrate knowledge on traits that provide natural immunity against malaria and protection afforded by such intervention studies.

1.2 INTRODUCTION

One of the most studied infectious diseases is malaria (Greenwood and Mutambingwa, 2002), which affects 500 million people each year and is a leading cause of death globally. Although malaria is prevalent in many areas of the world, Africa bears the biggest burden of this disease (Miller, 1994). Archaeological evidence suggests that malaria has had a significant effect on humans only in the past 10,000 years, which is consistent with the advent of agriculture, animal domestication, and increased human population densities in this geographic region (Livingstone, 1971).

Malaria is a febrile illness caused by a protozoa parasite of the genus *Plasmodium,* of which four species infect humans: *P. falciparum, P. vivax, P. ovale,* and *P. malariae.* The malaria parasites undergo a developmental cycle in the female anopheline mosquito,

which is the vector. The sporozoites are injected into the human host following a bite by the infected mosquito. The sporozoites rapidly migrate to the liver where they undergo a non-pathogenic pre-erythrocytic phase and then enter the RBC to commence the pathogenic erythrocytic phase. The parasites then continue their multiplicative asexual cycle. The erythrocytic phase is characterized by fever, chills and sweats (which vary in periodicity among the different species), enlargement of the spleen (spleenomegally), anemia, cerebral malaria and acid-base imbalance (Woodruff, 1978; Miller, 1985; Strickland, 1991, Marsh *et al.*, 1995).

P. falciparum causes the most severe form of malaria and is associated with the greatest degree of morbidity and mortality. It is spread by the female *anopheles* mosquito that is found in warm, moist climates and is distributed over most of tropical Africa, parts of India, Pakistan, South-east Asia including Indonesia and New Guinea, Central and South America (Greene, 1993).

During the course of human evolution in regions where malaria was prevalent, naturally occurring genetic defense mechanisms evolved to limit disease severity. The pathogenic stage of infection occurs in the RBC and is no wonder that all aspects of RBC including the Hb (hemoglobinopathies) such as sickle cell and thalassemias, membrane antigens (Duffy factor) and enzymes (G6PD) are involved in protection against malaria (Tishkoff *et al.*, 2001). Other natural forms of protection against malaria exist. These include the human leukocyte antigen (HLA) whose genes have been prominent candidates for

investigation as they have a remarkable degree of genetic polymorphism. Because the primary role of HLA molecules is to present peptides derived from infectious pathogens to T cells in the immune response against infection, the high degree of polymorphism of the HLA genes may have been attained and maintained through natural selection imposed by infectious organisms. In the case of malaria, the first convincing association study was carried out in West Africans, and the frequent *HLA-Bw 53* allele and the special DRB1*1302-DQB1*0501 haplotype were found to be independently associated with reduced susceptibility to severe malaria. Furthermore *HLA-Bw 53* restricted cytotoxic T lymphocytes are reported to recognize a conserved epitope in *P. falciparum* liver stage antigen type 1 (Hananantachai *et al.*, 2005).

Another form of natural protection against malaria is through the Tumor necrosis factors (TNF), which refers to a group of cytokines family. Since antibody formation is a prominent part of the host immune response against malaria parasite proliferation is associated with the generation of soluble plasmodial antigens. These antigens may be available for binding by reactive antibodies to form immune complexes (ICs). ICs can activate the complement cascade and stimulate macrophages to produce proinflammatory cytokines such as the TNF. These are a group of cytokines that can cause apoptosis, a type of programmed cell death (PCD), which involves an orchestral series of biochemical events leading to a characteristic cell morphology and death. The apoptotic process is executed in such away as to safely dispose of cell corpses and fragments (Mibei *et al.*, 2005).

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1.3 GLUCOSE-6-PHOSPHATE DEHYDROGENASE

G6PD is a "housekeeping enzyme" that performs vital functions within all cells of the body. However, within the erythrocyte, which lacks a nucleus, mitochondria, and other organelles, there are certain constraints on metabolism and this enzyme has a particularly important role. G6PD is important in maintaining cellular milieu in a reduced state. It does this by initiating a cascade of catalytic reactions that are summarized in Figure 1. G6PD catalyzes the first step of the pentose phosphate pathway (PPP) and a series of side reactions off the main glycolytic pathway (Figure 1).

G6PD initiates the glycolytic pathway by catalyzing the oxidation of glucose-6-phosphate to 6-phosphogluconate through the co-enzyme $NADP^+$, which is reduced to NADPH. The second enzymatic step is also associated with the reduction of $NADP^+$ to NADPH. The NADPH produced as a consequence of these reactions reduces oxidized glutathione (GSSG) to its reduced state (GSH) in a reaction catalyzed by glutathione reductase. GSH then reduces hydrogen peroxide, a powerful oxidant produced in the course of cellular metabolism and inflammatory responses to water, a reaction that is catalyzed by glutathione peroxidase (Newsholme and Leech, 1983; Beutler, 1983; WHO, 1989; Luzzatto and Mehta, 1989). NADPH is essential in RBCs to protect against the physiologically high levels of oxidative damage, which it does by maintaining a high level of reduced glutathione (GSH) in the cell to maintain a reducing environment.

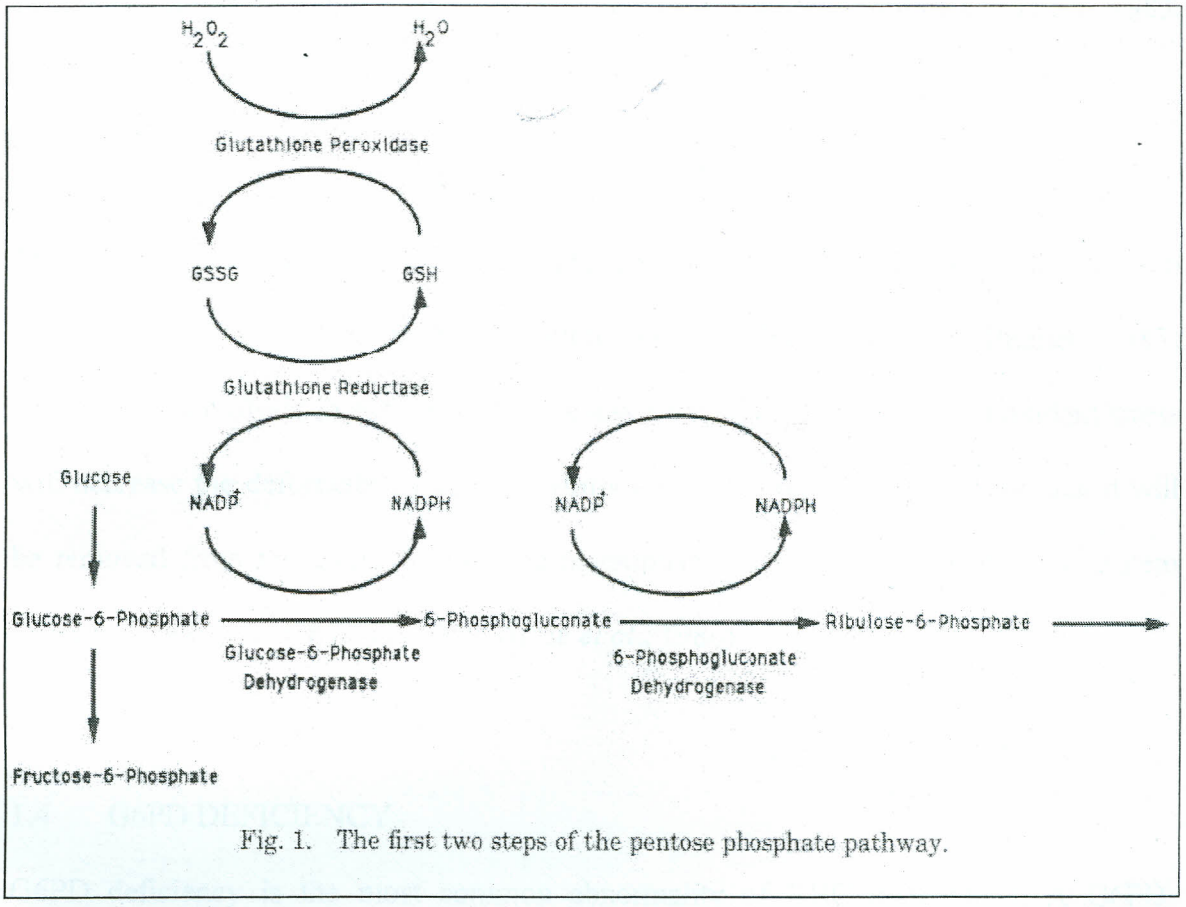

Fig. 1. The first two steps of the pentose phosphate pathway.

Figure 1: The first two steps of the pentose phosphate pathway (Greene, 1993)

The GSH protects the sulphydrl groups in hemoglobin and in the RBC membrane from oxidation. In the presence of oxidizing agents in the form of free radicals or peroxides the levels of GSH drops and can be restored by the action of glutathione reductase, which needs an adequate supply of NADPH (Filosa *et al.*, 2003).

The main function of the PPP is the generation of cellular reducing capacity through the production of NADPH and ultimately GSH. PPP is the only mechanism available to the RBC for generating reducing capacity and is thus essential for cell survival, while in

other cells, alternative means of NADPH production exist (Newsholme and Leech, 1983; Beutler, 1983; WHO, 1989).

In the normal RBC, uncompensated oxidant stress results in the oxidation of hemoglobin to methemoglobin, Heinz body formation, and membrane damage (Beutler, 1983) resulting in hemolysis in extreme cases. A less severe but uncompensated oxidant stress will decrease the deformability of the erythrocyte and increase the likelihood that it will be removed from the circulation by the macrophages of the reticuloendothelial system (Beutler, 1983; Johnson *et al.*, 1986; Arese *et al.*, 1986).

1.4 G6PD DEFICIENCY

G6PD deficiency is the most common abnormality of RBC metabolism. A G6PD deficient erythrocyte has only a limited ability to deal with an increased oxidant stress. G6PD deficient individuals may experience hemolysis (which at times is explosive and severe) under a variety of circumstances, which include ingestion of certain drugs or foods, exposure to certain chemicals, infection, or hypoxia (Belsey, 1973; Chevion *et al.*, 1982; Calabrese, 1984; Arese *et al.*, 1986; Luzatto and Mehta, 1989; Beutler *et al.*, 1989a). This hemolysis produces a significant degree of morbidity and mortality in G6PD deficient individuals. However, the increased vulnerability of the G6PD deficient RBC to oxidant stress is the mechanism underlying relative protection against *falciparum* parasitization.

G6PD deficiency occurs with increased frequency throughout Africa, Asia, the Mediterranean, and the Middle East (Figure 2). In the United States, black males are most commonly affected, with a prevalence of approximately 10 percent. Prevalence of the deficiency is correlated with the geographic distribution of malaria (Figure 2), which has led to the theory that carriers of G6PD deficiency may incur partial protection against malaria infection (Frank, 2005).

1.5 G6PD VARIANTS

G6PD is a cytoplasmic enzyme that is found in all cells of the body. The G6PD monomer consists of 515 amino acid residues with a calculated molecular weight of 59,256 daltons. The active enzyme exists as a dimer and contains tightly bound NADP. Aggregation of the inactive monomers into catalytically active dimers and higher forms requires the presence of NADP. Thus NADP appears to be bound to the enzyme both as a structural component and as one of the substrates of the reaction (Beutler, 1994).

G6PD B the wild genotype is found in most people worldwide and is identical to that of the chimpanzee and gorilla (Luzzatto and Battistuzzi, 1985). In populations outside the tropical and semitropical regions it is virtually the only G6PD isoenzyme, except for rare private mutations, that do not reach polymorphic frequencies (> 0.1%), (Luzzatto and Battistuzzi, 1985). Of all human loci, the G6PD locus is the most polymorphic.

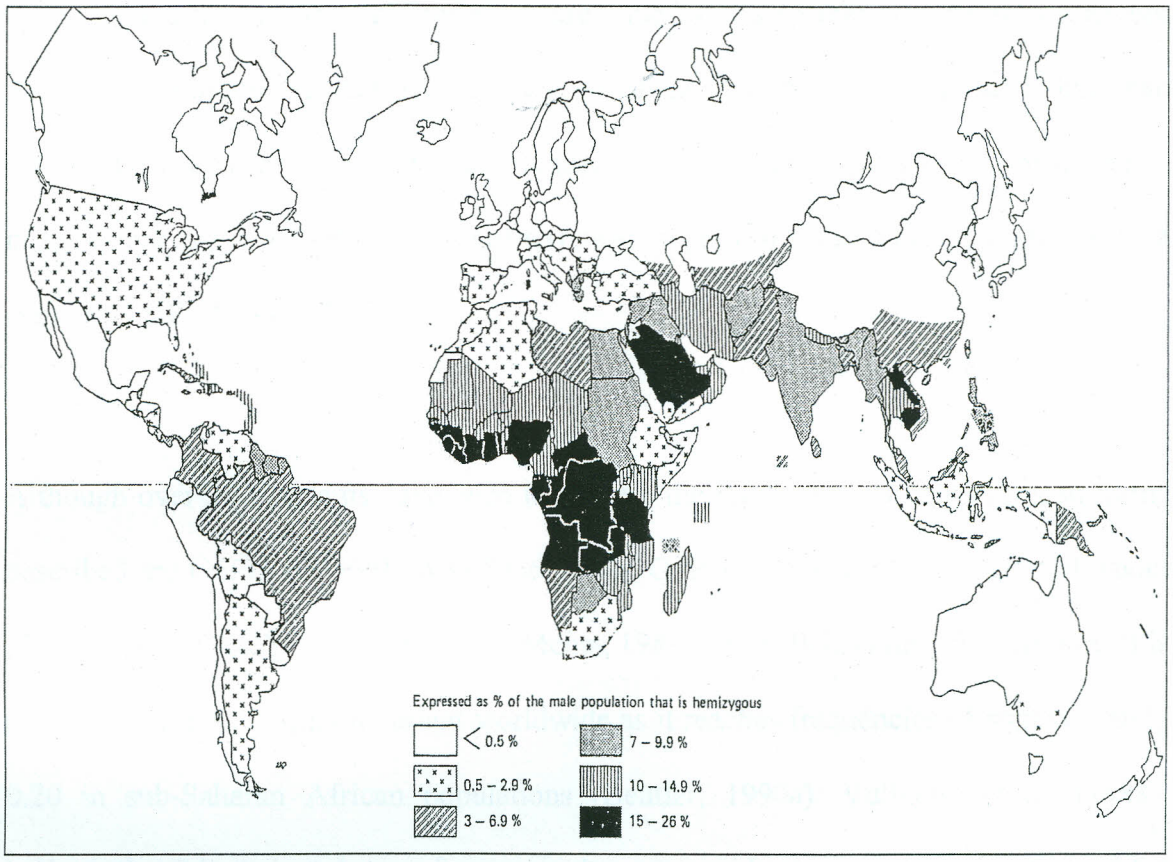

Figure 2: Map showing the global distribution of G6PD deficiency

The % values are the frequencies of G6PD-deficient males (because the G6PD gene is X-linked, these values are identical to gene frequencies, WHO Working Group, 1989

Over 300 variants have been fully described on the basis of biochemical and electrophoretic properties (Luzzatto and Battistuzzi, 1985; Luzzatto and Mehta, 1989; Beutler, 1990a). In their comprehensive review, Luzzatto and Mehta indicate that 77 of these variants have reached polymorphic frequencies (> 0.1%). Eleven of these polymorphic alleles have normal activity and 66 of the variants have decreased activity and are called deficient variants (Luzzatto and Mehta, 1989). It appears that total G6PD deficiency is incompatible with life. Thus most mutations are missense mutations and

deletions and are found in multiples of three nucleotides so that a frame shift does not occur. G6PD Mediterranean is a deficient variant with enzyme activity that is less than 5% of the wild type. It reaches gene frequencies of between 0.10 and 0.25 or higher in many populations of the circum-Mediterranean region and the Near and Middle East (Luzzatto and Mehta, 1989).

Although over 300 variants have been identified, the three most common African forms described are G6PD B, G6PD A and G6PD A-. G6PD A is a normal variant with about 90% activity of G6PD B (Luzzatto and Mehta, 1989). The allele coding for this variant is probably the most common variant worldwide as it reaches frequencies of approximately 0.20 in sub-Saharan African populations (Beutler, 1990a). Vulliamy *et al.* (1988), indicate that this variant derives from a single amino acid substitution of aspartic acid for asparagine at position 126, and that this is the result of an adenine to guanine substitution at nucleotide number 376 (Table 1). G6PD A- is a deficient variant with 8-20 % of the activity of G6PD B, which reaches allele frequencies of about 0.15 in sub-Saharan African populations (Luzzatto and Mehta, 1989). It appears that this variant has arisen from the G6PD A variant as both have aspartic acid substitutions at position 126. The G6PD A- variant has an additional guanine to adenine substitution at nucleotide 202 at the genetic level leading to a valine to methionine substitution at the protein level at position 68 (Beutler *et al.*, 1989b).

Table 1: Classification of enzyme variants by activity (Beutler, 1990b and 1994)

Four most common G6PD variants out of 300+ known		
Gd^{B}	Normal Activity	World wide distribution
Gd^{A}	Normal Activity: Guanine substituted for adenine at DNA position 376, Aspartic acid substituted for Asparagine at position 126	Africa (most common variant)
Gd^{A-}	8 - 20% Normal Activity and polymorphisms can occur in many areas: Adenine for guanine at DNA position 202 or guanine for thymine at position 680 or thymine for cytosine at position 968, Aspartic acid substituted for Asparagine at position 126, Methionine for Valine at position 68, Arginine substituted for Leucine at position 227 and Leucine for Proline at position 323.	Africa
Gd^{Med}	< 5% Normal Activity; Thymine for cytosine at DNA position 563, Phenylalanine for Serine at position 188.	Iran, Iraq, India, Pakistan, Greece, Sardinia

With development of the ability to define the mutations in the G6PD gene, the G6PD A- which had generally been regarded as a distinct, homogenous mutation, proved to be the result of the superimposition of several point mutations on the background of G6PD A (position 376 of the G6PD gene). The mutation found in G6PD A- is always in combination with G6PD A. In most cases, the second mutation affects nucleotide G at position 202 of the G6PD gene, but it can also affect G at position 680 leading to the amino acid change of Arginine for Leucine at position 227 or T at position 968 (Leucine substituted for Proline at position 323) (Beutler, 1994). The fact that African deficiency mutations of the G6PD A- type appear to occur only in the context of the 376G mutation of G6PD A suggested that the primordial human G6PD may have been G6PD A (Beutler *et al.*, 1989). However, a finding by Town *et al.* (1992) indicates that the 202A mutation produced by site-directed mutagenesis alone is not enough to produce enzyme deficiency: the 376G mutation which ordinarily alone does not produce enzyme deficiency, is required. Thus it is possible that the mutations at nucleotide 202, 680 and 968 would have no selective advantage against malaria when they occur in a G6PD gene that does not have the 376G mutation.

In the deficient variants, the mutation does not impair enzyme synthesis; rather the stability of the enzyme is affected. Accordingly, the enzyme activity is normal in reticulocytes of G6PD deficient individuals, but older red cells are markedly deficient. Exposure to oxidants therefore induces hemolysis of older red cells but not of younger ones. The recent elucidation of the crystal structure of G6PD has provided insights into

the basis of reduced stability. It appears that the mutations associated with disease result in a loss of normal folding of the G6PD protein. Apparently, the unfolded forms are susceptible to proteolytic degradation and therefore have a shorter half-life. Thus, G6PD deficiency, along with alpha-trypsin deficiency, belongs to a group of diseases in which defective folding of the proteins lies at the heart of the disorder (Saad, 2002).

1.6 G6PD DEFICIENCY AND HEMOLYTIC ANEMIAS

A G6PD deficient RBC has a limited ability to deal with oxidant stress and under certain conditions, hemolyses easily.

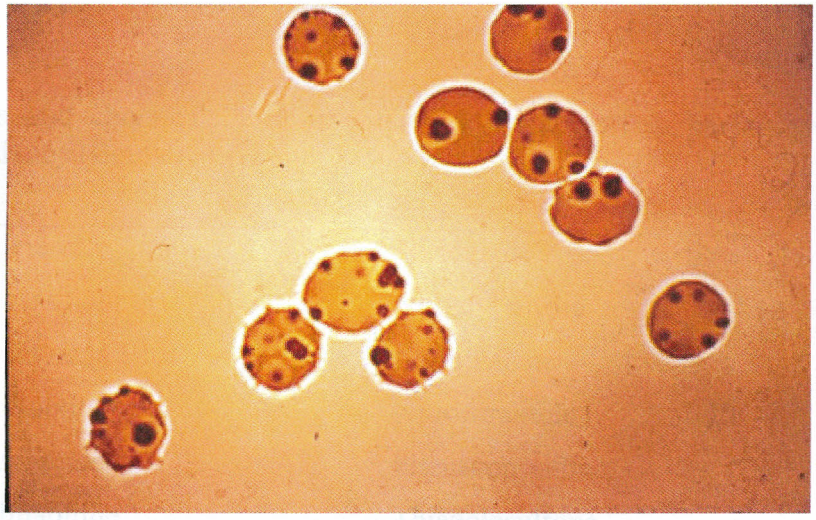

Figure 3: Heinz bodies

Source: (http://walkerma.wordpress.com/2007/03/05/word-of -the-week-heinz-bodies/)

1.6.1 *DRUG INDUCED HEMOLYSIS*

G6PD deficiency was discovered as a result of a series of investigations performed to understand why some persons were uniquely sensitive to the development of hemolytic anemia when they ingested the 8-aminoquinoline antimalarial drug, primaquine (Beutler, 1980). It was later discovered that primaquine is but one of the many drugs that shorten RBC life span in G6PD deficient persons (see Table 2). Exposure to such drugs is followed by a fall in the Hb concentration after a 1-2 day delay. Heinz bodies (Figure 3), particles of denatured protein adherent to the RBC membrane appear in the early stages of drug administration and disappear as hemolysis progresses (Dem *et al.*, 1954). Therefore it is prudent to test the effect of antimalarial drugs on individuals who are G6PD deficient before recommending their general use in the field.

Table 2: Drugs and chemicals that are linked to hemolysis in persons with G6PD deficiency (Beutler, 1978)

Acetanilid	Niridazole
Furazolidone	Isobutyl nitrite
Methylene Blue	Naphthalene
Nalidixic Acid	Nitrofurantoin
Naphthalene	Phenazopyridine
Primaquine	Thiazolesulfone
Sulfacetamide	Toluidine blue
Sulfamethoxazole	Trinitrotoluene
Sulfanilamide	Urate oxidase
Sulfapyridine	Phenylhydrazine

1.6.2 *FAVISM*

Favism is caused by ingestion of the fava bean, *Vicia faba.* This occurs in Gd^{Med} individuals, but occasionally favism has been observed in a patient with G6PD A- (Calabro *et al.*, 1989 and Galiano *et al.*, 1990). Pathogenesis of favism and drug-induced hemolytic anemia may be essentially the same (Arese *et al.*, 1990). Vicine, convice, ascorbate, and L-DOPA are abundant in fava beans and have been considered candidate toxins. The most likely offenders are vicine and convicine which are β-glycosides of pyrimidine compounds that are converted by β-glycosidases to their aglycones, vicine and isouramil, respectively. These compounds form reactive semiquinoid-free radicals and can generate active oxygen species. In G6PD deficient individuals, the reduced ability to contain oxidative stress results in the formation of ferrylhemoglobin, methemoglobin, and inactivation of various enzymes (Repine *et al.*, 1979; Davies *et al.*, 1987; Hochstein, 1988; Niki *et al.*, 1988; Rakitzis *et al.* 1989; Saltman, 1989; Arese *et al.*, 1990; Melhorn, 1991).

1.6.3 *INFECTION INDUCED HEMOLYSIS*

Many different types of infection, which include bacteria, viruses and rickettsia, may trigger hemolysis through unknown mechanisms. It has been suggested that active oxygen species that are discharged by leukocytes following phagocytosis are responsible for the damage of erythrocytes (Baehner et al., 1971).

1.6.4 *NEONATAL JAUNDICE*

This is a life and health threatening consequence of G6PD deficiency in infants. As RBC lyse, they release Hb. The released heme molecules are converted to bilirubin and become bound (as unconjugated also called indirect bilirubin) to serum and are transferred to the liver where they are conjugated to glucuronate by glucuronyl transferase. Conjugated (direct) bilirubin is excreted into bile. Impairment of the liver function due to enzyme deficiency may lead to bilirubin being retained in circulation causing jaundice (Beutler, 1994).

1.7 INHERITANCE OF G6PD

The genetic locus for G6PD in humans and all mammals is located on the telomeric region of the long arm of the X-chromosome (at Xq 28). Interestingly, this locus is flanked by 300 kb on each side by Factor VIII and the red/green color vision genes, which have been widely studied for their association with hemophilia (Toole *et al.* 1986) and color blindness, respectively (Nathans *et al.* 1986; Deeb *et al.* 1992). Because inheritance of G6PD deficiency is sex-linked, five genotypes are possible (Figure 3). In females, three forms can exist: Homozygous normal (Gd^B/Gd^B), heterozygous carrier (Gd^B/Gd^{A-}) and homozygous deficient (Gd^{A-}/Gd^{A-}). In the males the condition exists as hemizygous normal (Gd^B) or hemizygous deficient (Gd^{A-}), (Beutler, 1990b).

The frequency of the deficient condition is higher in males than females (Figure 3), since males, being hemizygous need only one copy of the allele to be fully deficient, while females need both deficient alleles (Greene, 1993). Hemizygous deficient males and

homozygous deficient females express the same degree of enzyme deficiency. Due to the random deactivation of one X-chromosome during embryological development in the female, heterozygous females actually have two populations of red cells (G6PD normal and G6PD deficient), a condition referred to as the Lyon Mosaic Effect (Beutler, 1983; Luzzatto and Battistuzi, 1985). Because of this, degree of G6PD activity in heterozygous females depends on the proportion of normal to deficient RBC. In most cases, the activity will be between 20 and 80% of the normal. However, a few heterozygotes (about 1%) may have only normal or almost only G6PD deficient cells (Lewis *et al.*, 2001)

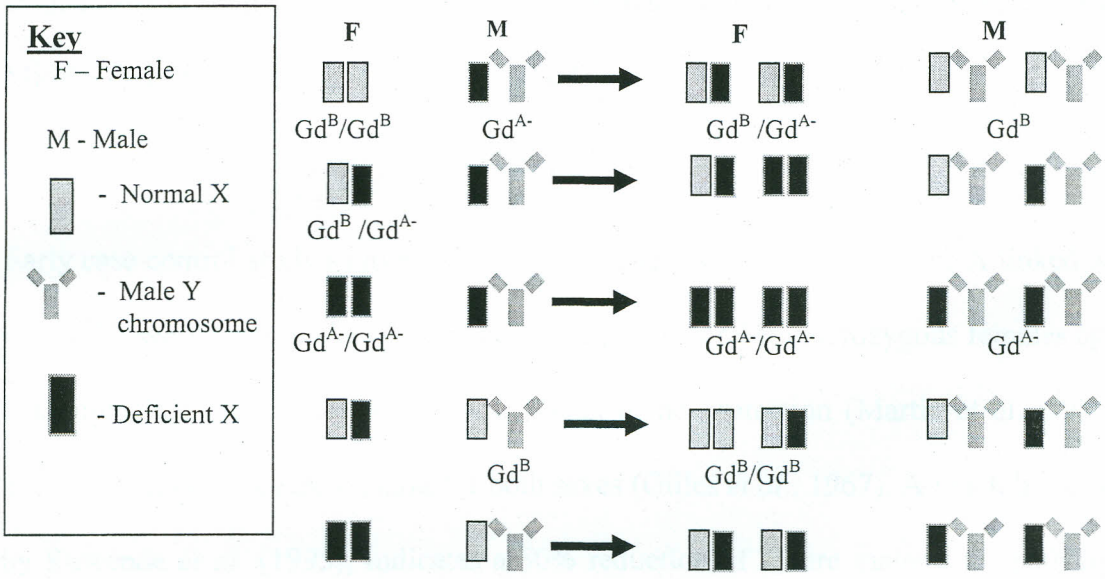

Figure 4: Five possible G6PD genotype inheritances

1.8 G6PD DEFICIENCY AND MALARIA PROTECTION

P. falciparum can invade erythrocytes of all ages though it preferentially attacks immature RBC (Cecil, 1992). This is a significant phenomenon since G6PD activity is markedly reduced in older erythrocytes. This renders mature erythrocytes more vulnerable to the oxidant stress and may explain preference of young erythrocytes by *P. falciparum* (Woodruff, 1978; Miller, 1985; Strickland, 1991).

The evidence supporting the increased resistance to malaria in G6PD deficient individuals has predominantly come from geographical distribution and case control studies. The frequency of this disorder is extremely high in tropical countries and the Middle East (reviewed in Mehta *et al.*, 2000).

Early case control studies have yielded conflicting results (because of the X linked nature of G6PD) with some studies observing either protection for heterozygous females against uncomplicated malaria (Bienzle *et al.*, 1972) or no protection (Martin et al., 1979), or protection against severe malaria for both sexes (Gilles *et al.*, 1967). A much larger study by Ruwende *et al.* (1995), indicates a 50% reduction of severe malaria for both female heterozygotes and male hemizygotes. Homozygous females probably have a similar level of protection from malaria, although this genotype is quite rare. This suggests that the selective advantage conferred by resistance to malarial infection is counterbalanced by a selective disadvantage associated with the enzyme deficiency.

The molecular basis of the protective effect has been investigated *in vitro*. While several studies show no inhibitory effect of G6PD mutations on parasite growth *in vitro* (Miller *et al.*, 1984), others have shown inhibition in short-term replication assays (Roth *et al.*, 1983). Under conditions of oxidative stress, which can cause major hemolysis in G6PD deficient individuals, growth of *Plasmodium* seems to be inhibited more consistently (Miller *et al.*, 1984; Golenser *et al.*, 1988). Recent studies by Cappadoro *et al.* (1998) have shown that G6PD deficient erythrocytes were more susceptible to phagocytosis by peripheral blood monocytes, particularly at the early ring stage of infection. This was concomitant with increased binding of IgG and complement C3 to infected erythrocytes.

1.9 RATIONALE OF STUDY

Western Kenya is a malaria holoendemic region and very little information on prevalence of G6PD deficiency is available. This study will determine the extent and possible implications of G6PD deficiency in the population living in the Kombewa Division in the Lake Victoria basin. Although over 300 variants are identified, only G6PD A and A- were investigated in this study, as they are the most common mutations occurring in Africa. Various interventional studies are planned against malaria in these populations and since G6PD deficiency is protective against malaria, it is important to unambiguously differentiate the protection from natural traits with that afforded by malaria vaccines or drugs.

1.10 OBJECTIVES OF STUDY

1.10.1 *GENERAL OBJECTIVE*

i) The aim of this study to determine the prevalence of G6PD deficiency in children targeted to receive a malaria vaccine.

1.10.2 *SPECIFIC OBJECTIVE*

i) To determine the gene frequencies of G6PD deficiency using a combination of PCR and RFLP (alleles B, A and A-) in the study population.

ii) To determine whether the G6PD locus in the studied population was in Hardy-Weinberg equilibrium.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 STUDY SITE AND DESIGN

The study catchment area was Kombewa division within the Lake Victoria Basin of Western Kenya (Figure 4). Blood samples were collected once from children presented for screening and potential enrollment for malaria vaccine trial. A total of 570 children of ages 12-47 months were screened, of which 400 were enrolled to receive either the malaria vaccine or comparator article.

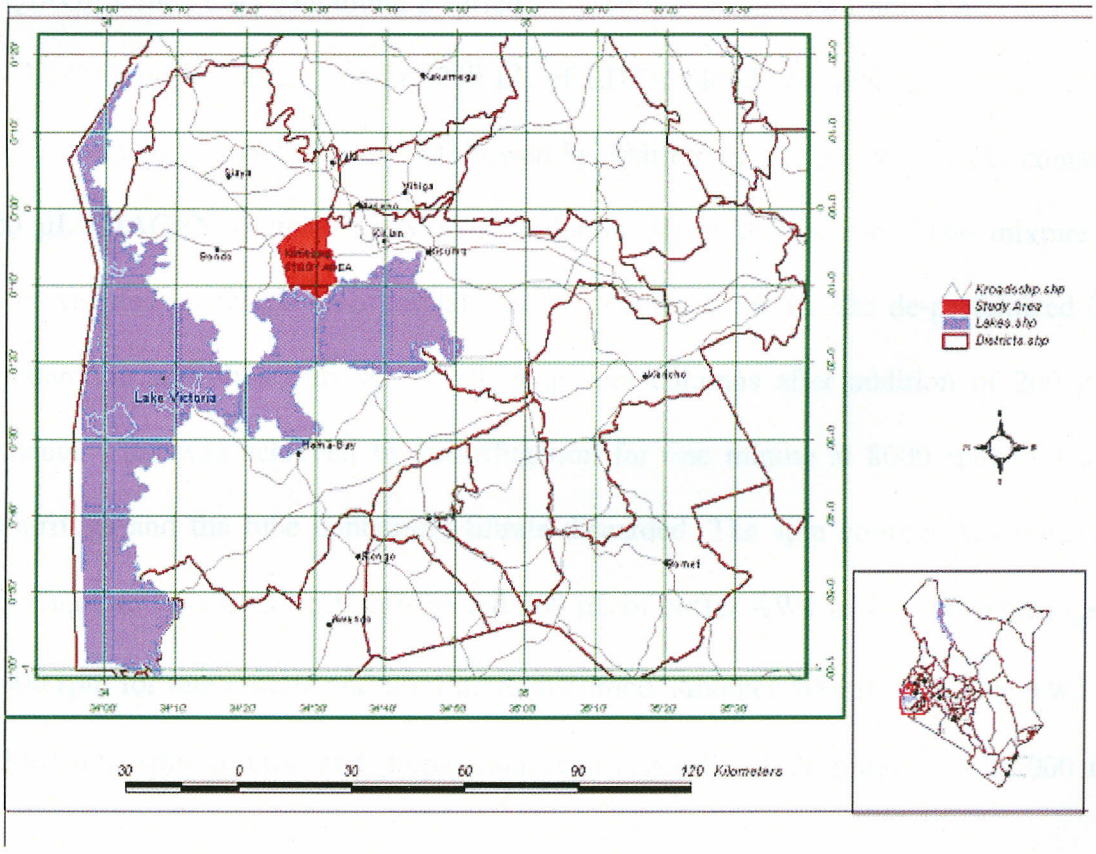

Figure 5: Map of study area showing Kombewa Division highlighted in red Source: http://www.maplandia.com/kenya/western

2.2 ETHICAL CONSIDERATION

The study was voluntary and parents or guardians signed voluntary consent document to allow participation of their children. Ethical clearance for this study was obtained from KEMRI ethical committee.

2.3 DETERMINATION OF G6PD GENOTYPES

2.3.1 *ISOLATION OF GENOMIC DNA*

The DNA was isolated from EDTA blood using QIAamp DNA Blood mini Kits (QIAGEN Inc., CA) containing a variety of propriety wash (AW1 and AW2) and elution (AE) buffers. In this procedure, 200 µL of EDTA blood was placed in sterile 1.5 mL micro centrifuge tubes. This was followed by addition of 200 µL lysis buffer containing 20 µL QIAGEN protease enzyme in order to digest the proteins. The mixture was vortexed and incubated on a hotplate for 10 minutes at 56 °C. The de-proteinized DNA preparation was then purified on QIAamp spin columns after addition of 200 µL of ethanol. This was achieved by centrifugation for one minute at 8000 rpm in a micro centrifuge and the tube containing filtrate discarded. The spin column was transferred into another 1.5 mL collection tube and 500 µL of buffer AW1 added and centrifuged at 8000 rpm for one minute and the filtrate discarded. Another 500 µL of buffer AW2 was added into spin column and preparation centrifuged for three minutes at 14,000 rpm.

Finally, the purified DNA was eluted from the spin column by addition of 200 µL elution buffer (AE) and stored at –20 °C until required.

2.3.2 *PCR AMPLIFICATION*

PCR primers for amplification of the fragments, which include sites of 376, A→ G, 202 G→A, 680 G→T and 968 T→C mutations were as described in Samilchuk *et al.* (1999), and Beutler *et al.* (1989), and are shown in table 3.

Table 3: Primer sequences and specific annealing temperature for detection of G6PD gene mutations

Mutation	**Primer (F-R)**	**PCR annealing temperature, °C**
376 A→ G	F: 5 - CCCAGGCCACCCCAGAGGAGA - R: 5 - CGGCCCCGGACACGCTCATAG -	58
202 G→ A	F: 5 - CCACCACTGCCCCTGTGACCT - 3 R: 5 - GGCCCTGACACCACCCACCTT - 3	65
680 G→T	F: 5 - ACATGTGGCCCCTGCACCAC - 3 R: 5 - GTGACTGGCTCTGCCACCCTG- 3	69
968 T→C	F: 5 - TCCCTGCACCCCAACTCAAC - 3 R: 5 - CCAGTTCTGCCTTGCTGGGC - 3	65

The PCR mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 50 µM of each dNTP, 0.75 units of *Taq* polymerase (Applied Biosystems, Roche), 0.4 µM of each primer and 2 µL of genomic DNA in a total volume of 25 µl. The reactions were performed in a DNA thermocycler (Tetrad PTC-225 MJ Research Inc.), and programmed as follows: Initial denaturation step at 94°C for 10 min, followed by a total of 30 amplification cycles comprising: denaturation at 94°C for 45s, appropriate annealing temperature for the primers as indicated in (Table 3) for 45 seconds and a final extension time for 7 min at 72°C. Following completion of the PCR reaction, 5 µL of the amplicon was run on 2 % NuSieve agarose gel containing 5 µg/mL ethidium bromide and visualized on a UV transilluminator in order to verify successful amplification. The amplicons were stored at -20^{0}C for subsequent analysis by RFLP.

2.3.3 *RESTRICTION ENZYMES AND FRAGMENTS RESULTING FROM ENZYMATIC DIGESTION*

For the RFLP analysis, 10 µL of the PCR products were digested under the following conditions using the appropriate endonucleases as shown in Table 4: 37 °C for 3-16 hours using 1X NE Buffer (20 mM Tris-acetate, 50 mM Potassium acetate, 10 mM Magnesium acetate, 1 mM dithiothreitol supplemented with 100 µg/ml bovine serum albumin). Positive controls for each digest included samples from a normal female (Gd^B /Gd^B) or Male (Gd^B), heterozygous female (Gd^B/Gd^A or Gd^B/Gd^{A-}), homozygous female (Gd^A/Gd^A or Gd^{A-}/Gd^{A-}) or hemizygous male (Gd^A or Gd^{A-}). Products were analyzed by electrophoresis in a 2% NuSieve agarose gel containing 5 µg/ml ethidium bromide and visualized on a UV transilluminator

Table 4: Restriction enzymes and fragments resulting from enzymatic digestion

Mutation	**Restriction enzyme**	**Digested (Normal fragment), bp**	**Digested (Mutant fragment), bp**
376 A→ G	*Fok*I	308	125,183
202 G→A	*Nla*III	211	81,130
680 G→T	*BstN*I	213,29	29,98,115
968 T→C	*Nci*I	282	120,162

2.4 SAMPLE SIZE CALCULATION

The sample size was calculated from the formula:

$$n = \left[\frac{z_{\alpha/2}\sigma}{E}\right]^2$$

Where n = sample size

$z_{\alpha/2}$ = is known as the critical value, the positive z value that is at the vertical boundary for the area of $\alpha/2$ in the right tail of the standard normal distribution.

σ = the population standard deviation

E = Margin of error (http://www.isixsigma.com/library/content/c000709a.asp)

The sample size calculation was based on clinical malaria attack rate in a longitudinal cohort study in the area of study that showed monthly clinical malaria attack rates of

between 20 and 55% in children aged one to three years. The sample size calculation for the malaria vaccine trial assumed a 50% attack rate over the six-month follow-up period with time to clinical malaria following an exponential distribution. A trial with 200 subjects per study arm would have 80% power to detect a 30% reduction in the six-month cumulative incidence rate.

2.5 DATA ANALYSIS:

Data was processed and stored in Microsoft Access 2000. Data cleaning and statistical analyses using proc means and proc freq were performed in SAS versions 9.13 (2003-2005, SAS Institute, Inc., Cary, North Carolina, USA). The genotypic and allele frequencies were calculated. Separate analysis was done for females and males due to the fact that the G6PD deficiency is X-linked. To determine if the distribution of these alleles in the population were in Hardy-Weinberg equilibrium, the chi-square test was used to test independence between the expected and the observed values: the expected values were manually calculated.

2.5.1 *DETERMINATION OF WHETHER OBSERVED GENOTYPE FREQUENCIES ARE IN HARDY-WEINBERG EQUILIBRIUM*

To determine whether the observed genotype frequencies were significantly different from the expected, the Hardy-Weinberg equilibrium equation ($p^2 + 2pq + q^2 = 1$) was used to first calculate the probable genotype frequencies. Chi square test was then used to determine whether the observed and the expected genotype frequencies were statistically different. In this equation, p is defined as the frequency of the dominant alleles (Gd^B and

Gd^A) and q as the frequency of the recessive allele (Gd^{A-}). The female homozygous gene frequency was used to calculate the expected dominant and heterozygous genotype frequencies in the population. The females were used since, unlike the males, they have 2 X-chromosomes in which the G6PD gene resides.

Thus:

p^2 = Predicted frequency of dominant Gd^B/Gd^B, Gd^B/Gd^A and Gd^A/Gd^A

2pq = Predicted frequency of Gd^A/Gd^{A-} and Gd^B/Gd^{A-}

q^2 = Predicted frequency of Gd^{A-}/Gd^{A-}

In this equation ($p^2 + 2pq + q^2 = 1$):

$$p = BB + ½BA-$$

Likewise, q equals all of the alleles in individuals who are homozygous recessive (A-A-) and the other half of the alleles in people who are heterozygous (BA-).

$$q = A-A- + ½\ BA-$$

Because there are only two alleles in this case, the frequency of one plus the frequency of the other must equal 100%, which is to say

$$p + q = 1$$

Since this is logically true, then the following must also be correct:

$$p = 1 - q$$

The Chi square test was then used to determine whether the observed and the expected individuals were statistically different. A null hypothesis (H_0) was set, stating that there

was no significant difference between the expected and the observed, therefore the population is in Hardy-Weinberg equilibrium. The chi-square was calculated using the equation below,

$$\chi^2 = \frac{\Sigma\,(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

This equation gives a critical value and referring to the Chi square table of critical values, the probability that the difference found is by chance alone is determined.

CHAPTER THREE

3. RESULTS

3.1 IDENTIFICATION OF Gd^B, Gd^A and Gd^{A-} VARIANTS OF G6PD

A total of 570 individuals (300 males and 270 females) who presented for screening were genotyped for identification of Gd^B, Gd^A and Gd^{A-} variants by a combination of PCR and RFLP. The amplification by PCR produced the expected product sizes (308 bp for the 376 A→G mutation, 211 bp for the 202 G→A mutation, 242 bp for 680 G→T mutation and 282 bp for the 968 T→ C mutation see table 4). On digestion with *Fok*I, PCR products from individuals with the wild type G6PD B genotype (Gd^B/Gd^B for females or Gd^B for males) did not digest (Figure 6, lane 2).

Females with a mutation on one chromosome (heterozygous Gd^B/Gd^A) were identified by presence of the wild type fragment size (308 bp) and two small fragments of 125 bp and 183 bp respectively (lane 3). The hemizygous males (Gd^A) or homozygous females (Gd^A/Gd^A) were identified (lane 4) by complete digestion of the amplicons into fragments of 125 bp and 183 bp. All samples identified as having a 376 A→G mutation (heterozygous and homozygous females and hemizygous males) were categorized as G6PD A and were then digested with *Nla*III enzyme. PCR products with the G6PD A genotype and lacking the 202 G→A mutation did not digest with *Nla*III enzyme (lane 5). Females with a mutation on one chromosome (heterozygous Gd^A/Gd^{A-}) were identified

by presence of the wild type fragment size (211 bp) and two small fragments of 81 bp and 130 bp (lane 6).

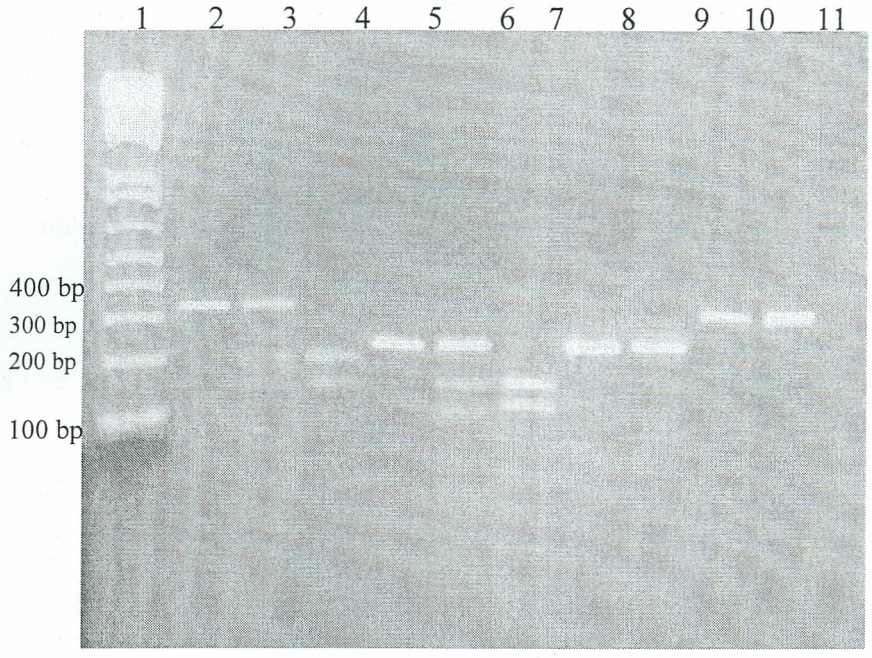

Figure 6: A representative photograph for RFLP of PCR products

RFLP of PCR products digested with FokI for 376 A→G mutation, NlaIII for 202 G→A mutation, BstNI for 680 G→T mutation and NciI for 968 T→C mutation. Lane 1, 1 Kb plus Molecular weight marker. **Fok1 digests showing**: Lane 2, a 308 bp product indicating the absence of FokI site in a homozygous normal female (GdB /GdB) or Male (GdB). Lane 3, the normal 308 bp product and two small fragments of 125 bp and 183 bp indicating the heterozygous female (GdB/GdA). Lane 4, two small fragments (125 and183 bp) indicating the homozygous female (GdA/GdA) or hemizygous male (GdA). **NlaIII digests showing:** lane 5 with a 211 bp fragment indicating the absence of NlaIII site in a homozygous female (GdA/GdA) or male (GdA) while lane 6, a 211bp normal product and two small fragments of 81 bp and 130 bp indicating heterozygous female (GdA/GdA- or GdB/GdA-), lane 7 with two small fragments of 81 bp and 130 bp indicating homozygous deficient female (GdA-/GdA-) or hemizygous deficient male (GdA-). **BstNI digests showing**: Lanes 8 and 9 with two bands of 29bp and 213bp indicating the absence of BstNI site in a homozygous female (GdA/GdA) or male (GdA). **NciI digests showing**: lane 10 and 11 with 282bp fragment indicating the absence of NciI site in a homozygous female (GdA/GdA) or male (GdA).

The hemizygous deficient males (Gd^{A-}) or homozygous deficient females (Gd^{A-}/Gd^{A-}) were identified (lane 7) by complete digestion of the amplicons into fragments of 81 bp and 130 bp. No 680 G→T or 968 T→C mutations were identified as shown by lanes 8 and 9 that have 29 bp and 213 bp fragments after digestion with *BstN*I for identification of 680 G→T mutation and lanes 10 and 11 showing a 282 bp fragment after digestion with *Nci*I for identification of 968 T→C mutation.

To differentiate the female heterozygous Gd^{B}/Gd^{A-}, Gd^{A}/Gd^{A-} and female homozygous Gd^{A-}/Gd^{A-}, digestion with *Fok*I and *Nla*III, (Figure 7, lane 2 and lane 5) showed that only one X-chromosome carried the 376 A→G mutation as well as the 202 G→A and this was identified as Gd^{B}/Gd^{A-}. The Gd^{A}/Gd^{A-}, upon digestion with *Fok*I yielded completely digested fragments (lane 3) meaning that both X-chromosomes carried the 376 A→G mutation, while digestion with *Nla*III (lane 6) showed that the 202 G→A mutation was carried on one X-chromosome. The Gd^{A-}/Gd^{A-} showed completely digested fragments with both *Fok*I (Lane 4) and *Nla*III (Lane 7) enzymes respectively. This meant that both X-chromosomes carried the 376 A→G mutation and the 202 G→A.

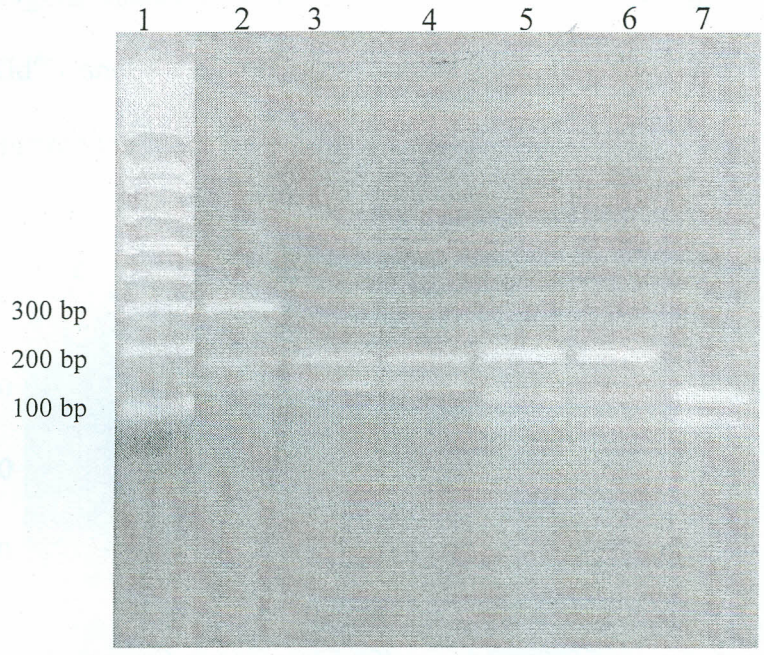

Figure 7: A photograph showing RFLP of PCR products for the differentiation of the female heterozygous Gd^{B}/Gd^{A-}, Gd^{A}/Gd^{A-} and the female homozygous Gd^{A-}/Gd^{A-}.

*Fok*I and *Nla*III digests for 376 A→G mutation and for 202 G→A mutation respectively. Lane 1, 1Kb plus Molecular weight marker, *Fok*I digests showing: lane 2, heterozygous female Gd^{B}/Gd^{A}, lanes 3 and 4, homozygous female (Gd^{A}/Gd^{A}). *Nla*III digests showing lane 5, heterozygous female (Gd^{B}/Gd^{A-}), lane 6, heterozygous female (Gd^{A}/Gd^{A-}) and lane 7 homozygous deficient female (Gd^{A-}/Gd^{A-}).

3.2 G6PD GENOTYPE FREQUENCIES

Of the 570 DNA samples examined, 338 (59.3 %) had the normal G6PD B genotype of which 209 (36.7 %) were hemizygous normal males (Gd^{B}) and 129 (22.6 %) were females (Gd^{B}/Gd^{B}). 153 (26.8 %) had the G6PD A genotype of which 59 (10.3 %) were males (Gd^{A}), 80 (14.0 %) were heterozygous females (Gd^{B}/Gd^{A}) and 14 (2.5 %) were homozygous females (Gd^{A}/Gd^{A}). 79 (13.9 %) had a combination of 376 A→G mutation and 202 G→A mutation that defines the G6PD A- genotype. Of these, 32 (5.6 %) were

hemizygous males (Gd^{A-}), 41 (7.2 %) were heterozygous females (Gd^{A}/Gd^{A-} and Gd^{B}/Gd^{A-}) and 4 (1.1 %) were homozygous females (Gd^{A-}/Gd^{A-}). These results are summarized in Figure 8.

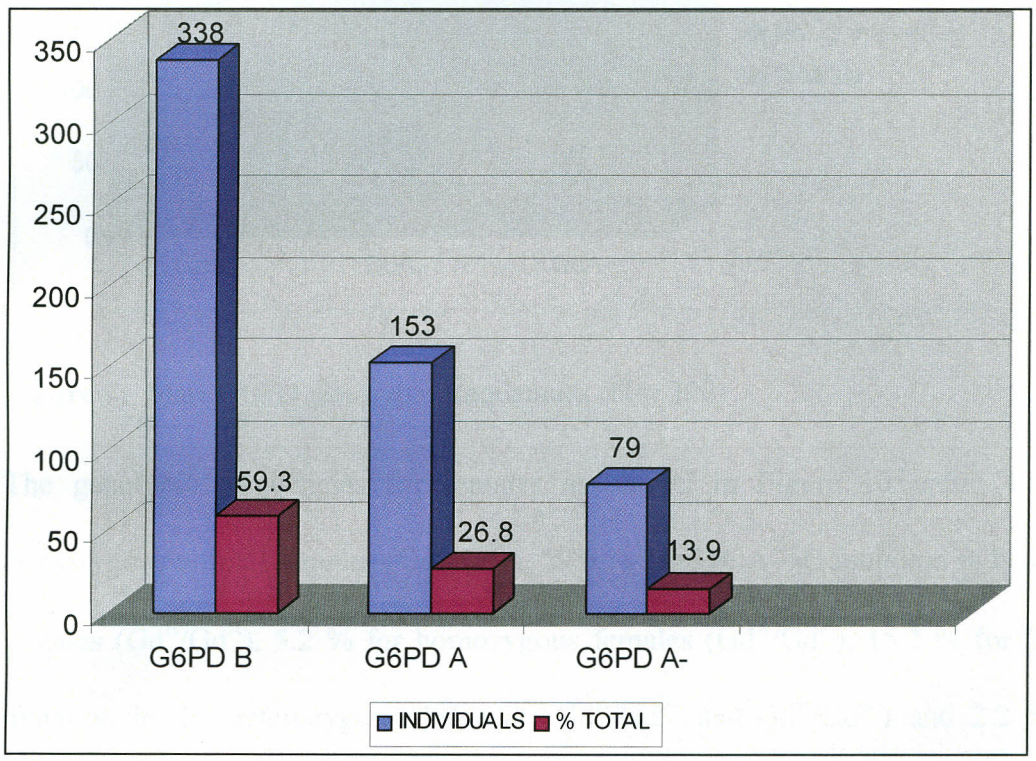

Figure 8: The total genotype frequencies in the population

Genotypic frequencies for males and females were calculated separately since inheritance of G6PD deficiency is X-linked. As shown in Figure 9, the genotypic frequencies for males were GdB, GdA (376 A→G mutation) and GdA- (202 G→A mutation), were 69.6 %, 19.7 % and 10.7 % respectively.

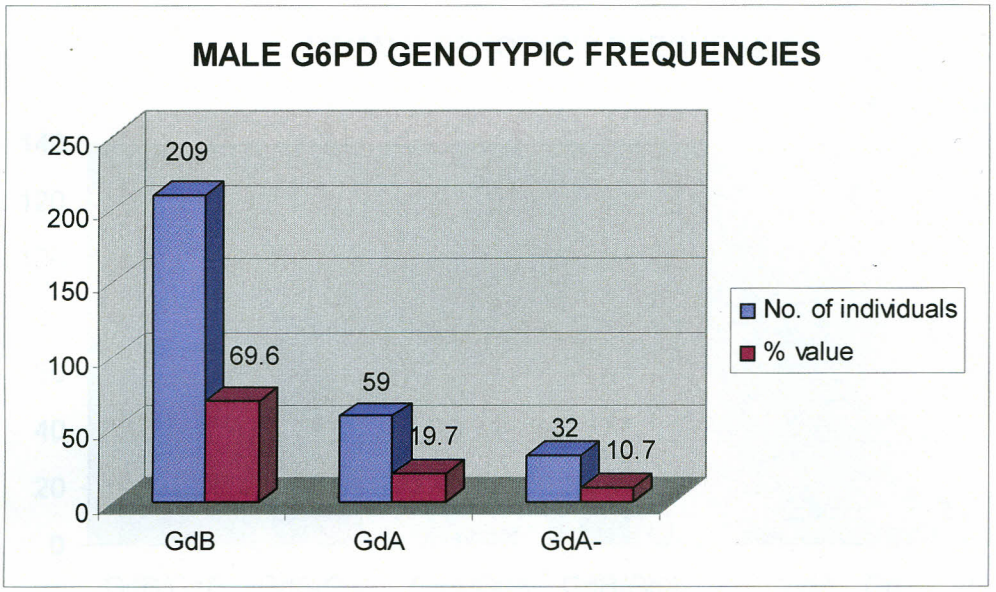

Figure 9: Male G6PD genotypic frequencies (N = 300)

The genotypic frequencies for females as shown in Figure 10 were: 47.8 % for homozygous normal females (Gd^B/Gd^B), 29.6 % for 376 A→G mutation in heterozygous females (Gd^B/Gd^A), 5.2 % for homozygous females (Gd^A/Gd^A), 15.2 % for 202 G→A mutation in the heterozygous deficient (Gd^A/Gd^{A-} and Gd^B/Gd^{A-}) and 2.2 % for the homozygous deficient (Gd^{A-}/Gd^{A-}).

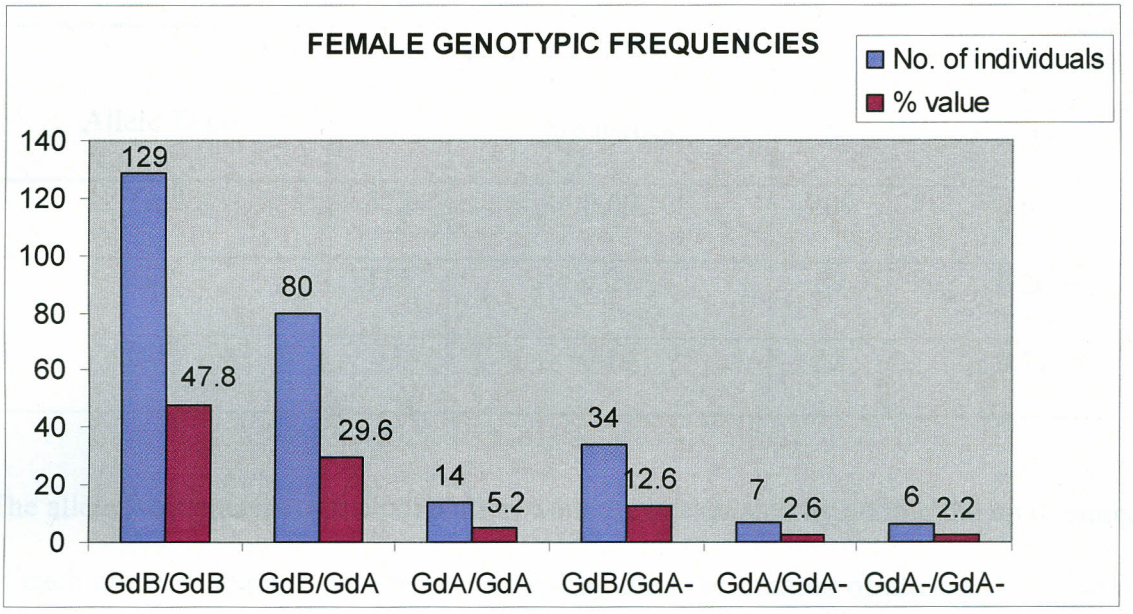

Figure 10: Female G6PD genotypic frequencies (N = 270)

3.3 G6PD ALLELIC FREQUENCIES

The G6PD gene is found in the X-chromosome therefore, males have one copy and their genotypic frequencies are same as the allele frequencies. Because females have two X chromosomes, the calculation of allele frequencies was different. For example, the frequency of allele B was calculated as follows: [2 x number of female (Gd^B/Gd^B)] + [1 x number of female (Gd^B/Gd^A)] / [total number of alleles]. Thus, the frequency of allele B is: [2 x 129] + [80] / [540] = 0.69. The same method was used to calculate the allele frequencies for Gd^A and Gd^{A-}, which were 0.21 and 0.10 respectively. The male and female allele frequencies are summarized in Table 5.

Table 5: Allele Frequencies by Gender

Allele Type	Female (N=540)		Male (N=300)	
	n	Frequency	n	Frequency
Gd^B	372	0.69	209	0.69
Gd^A	115	0.21	59	0.20
Gd^{A-}	53	0.10	32	0.11

The allele frequencies for the population were then calculated by adding the total number of each allele in females and males divided by the total number of alleles. As shown in Table 6, the population allele frequencies were 0.69, 0.21 and 0.10 for Gd^B, Gd^A and Gd^{A-} respectively.

Table 6: Total Allele Frequencies

Allele Type	Total Alleles = 840	
	N	Frequency
Gd^B	581	0.69
Gd^A	174	0.21
Gd^{A-}	85	0.10

3.4 DETERMINATION OF HARDY-WEINBERG EQUILIBRIUM

The observed homozygous allelic frequency (q) was 0.10 (Table 6).

Given that $q = 0.10$

Then $p = 1 - 0.10 = 0.9$, so $p^2 = (0.9)^2 = 0.81$

$2pq = 2(0.10)(0.9) = 0.18$

$q^2 = (0.10)^2 = 0.01$

To convert the expected genotypic frequencies into number of females in the population, the frequencies were multiplied by the number of females analyzed (270) and results tabulated as shown in Table 7.

Table 7: Female genotype expected and observed numbers

Variant	Expected	Observed
B/B	218	223
A/A-	49	41
A-/A-	3	6
Total	270	270

The χ^2 calculations based on Table 7 yields:

$$\frac{(223-218)^2}{218} + \frac{(41-49)^2}{49} + \frac{(6-3)^2}{3} = 0.12 + 1.4 + 3.0 = 4.52$$

From the Chi square table, at $P = 0.05$, the critical value is 3.84 at 1 degree of freedom. The 1 degree of freedom was arrived at from the equation:

df =k-1-m

Where k= the number of classes (genotypes)

m = the number of independent values we calculate from the data (allele frequencies)

Hence df = 3-1-1 = 1.

Since the calculated critical value (4.52) is greater than 3.84, there is only a 5 % probability that the difference between the observed and expected values is due solely to chance. The null hypothesis was rejected, and a conclusion made that there was a significant difference between the expected and the observed, therefore, the G6PD locus in the population is not in Hardy-Weinberg equilibrium.

CHAPTER FOUR

4. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 DISCUSSION

The data generated in this study shows a high prevalence (13.9 %) of the common African form of G6PD deficiency, namely G6PD A-, either in its severe form in hemizygous males (5.6 %) or homozygous females (1.1 %), or in the less severe heterozygous form (7.2 %) in females. As expected, the G6PD G→A mutation at position 202 occurred in combination with the 376 A→ G transition (Figure 6).

The prevalence of G6PD A genotype was 26.8 %, of which 59 (10.3 %) were males (Gd^A), 80 (14.0 %) were heterozygous females (Gd^B/Gd^A) and 14 (2.5 %) were homozygous females (Gd^A/Gd^A). On its own, the 376 A→G mutation, whether in hemizygous, homozygous or heterozygous forms, does not lead to loss of enzyme activity (Beutler, 1990b). A Gd^A gene frequency of 21 % (Table 7) was observed, which is comparable to the expected gene frequency of 20-30 % in Africa (Beutler, 1996). Similar prevalence range has been reported in Ivory Coast (0.28) (Coulibaly *et al.*, 2000) and Senegal (0.20) (Araujo *et al.*, 2006).

When originally identified, the G6PD A- variant was thought to be a single homogenous mutation, but it is now known to be a composite variant involving both the 376 A→G transition and the 202 G→A mutation, together constituting G6PD A-. The missense mutation 376 A→G by itself causes asymptomatic variant G6PD A with normal enzyme

activity (Takizawa *et al.*, 1987). Although the 202 G→A mutation is always found in combination with the 376 A→G (Beutler E., 1989), a recent study described a G6PD deficient individual with 202 G→A mutation (Hirono *et al.*, 2002). An in vitro study (Town et al., 1992) using recombinant human G6PD mutants expressed in *Escherichia coli* suggested that 202 G→A, as well as 376 A→G, do not cause enzyme deficiency when they occur independently and that the synergistic action of these two mutations is necessary to produce the G6PD A- phenotype. This synergistic interaction was also supported by the fact that Val 68 and Asn 126 are closely located in a 3-dimensional model of human G6PD (Naylor et al., 1996). Other variations of the G6PD A- deficiency involve the 680 G→T or the 968 T→C mutations in combination with 376 A→G (Beutler, 1996). These deleterious variants were not detected in the current study. In a study conducted in Senegal, the 376G/968C genotype was predominant with an overall prevalence of 25 % (Araujo *et al.*, 2006). The fact that the G6PD A- genotype occurs in the context of 376 A→G mutation was originally thought to indicate that the G6PD A was the primordial human G6PD variant. It is now known that G6PD A mutation occurred more recently compared to the prototype G6PD B (Beutler, 1994).

In this study, 13.9 % (Figure 8) of the individuals studied had a combination of 376 A→G mutation and 202 G→A mutation that defines the G6PD A- genotype. Of these, 5.6 % were hemizygous (Gd^{A-}) males, 7.2 % were heterozygous females (Gd^{A}/Gd^{A-}) and 1.1 % were homozygous females (Gd^{A-}/Gd^{A-}). Hemizygous deficient males and homozygous deficient females express the same degree of enzyme deficiency, while

hemizygous normal males and homozygous normal females also have comparable enzyme levels (Greene, 1993). Due to the random deactivation of one X-chromosome during embryological development, heterozygous females have two RBC populations one with normal enzyme activity and the other with activity that is as deficient as that seen in deficient hemizygous males. This phenomenon is referred to as the Lyon Mosaic Effect (Beutler, 1983; Luzzatto and Battistuzi, 1985). Mary Frances Lyon, an English geneticist, focused on the effects of radiation and other agents on genetic mutation, as well as the process of mutation itself and its applications to medicine. In 1961 she described inactivation of the X chromosome, the Lyon hypothesis, explaining how X chromosomes can sometimes be inactive in mammals (Oakes, 2002). Therefore, the proportion of RBCs with normal or deficient activity can be very variable (Beutler, 1994).

The overall genotypic frequency of G6PD A- found in the present study (13.9 %) is comparable to that observed in Uganda (8 %) (Sunil *et al.*, 2004) and Senegal (12 %) (Araujo *et al.*, 2006). However, it was much lower than that reported by Ruwende *et al.* (20 %) in a study conducted at Kilifi, Kenya and other tropical countries: 22 % in Ivory Coast, 24 % in Nigeria (Ademowo & Falusi, 2002). This is unexpected considering that transmission intensity of malaria in the Kenyan Coast is less intense compared to the Lake Victoria region and as such, the allele would be expected to be under reduced selection pressure. Because Lake Victoria basin has one of the highest malaria transmission intensity in the world (Lindblade *et al.*, 2004), the lower frequency of G6PD deficiency suggests that selection pressure might not be the only reason for the allele's

frequency. It is however not clear why Lake Victoria basin would have lower prevalence of G6PD deficiency. Possibly, it might be due to presence of other natural forms of protection such as the sickle cell trait and thalassemias, which are not as deleterious as G6PD deficiency and have a more protective advantage against malaria. In addition, since this study was carried out in a malaria holoendemic region, the G6PD deficient individuals might have been exposed to over-the-counter malarial drugs that possibly triggered hemolytic crisis causing deaths assumed to be malaria related.

Allele frequency for Gd^{A-} was 0.10 and comparable to that reported in Senegal (0.11) (Araujo *et al.*, 2006). It has however been reported to be much higher in Ivory Coast (0.21) (Coulibaly *et al.*, 2000) and Congo (0.22) (Bouanga *et al.*, 1998.

A recent study in Western Kenya (Moormann *et al*, 2003) showed a much lower prevalence of G6PD deficiency (7 %) compared to the 13.9 % in the current study. It is speculated that this discrepancy is attributable to the considerable overlap of heterozygote activities in part due to random X-chromosome inactivation. The quantitative method of detecting G6PD activity such as the fluorescent spot test used in Moormann's study is less reliable in identifying heterozygote females (Beutler, 1979). This quantitative assay measures the reduction of NADP to NADPH in the presence of glucose-6-Phosphate and hemolysate. The test is positive if the blood spot fails to fluoresce under ultraviolet light (Gregg *et al.*, 2000). The most accurate method for heterozygote detection is DNA analysis (Beutler, 1994) as used in the current study. Quantitative G6PD detection

methods also have limitations in accurately identifying deficiencies in G6PD deficient individuals undergoing a hemolytic crisis. In such a crisis, the older RBC population is selectively removed leaving the younger cells that have near-normal activity. G6PD screening test may give normal results, at least for a week or two after the hemolytic episode. The added advantage of DNA analysis is that the results are neither confounded by the presence of young erythrocytes or transfused cells (Beutler, 1994). In the studied population, the 376G/202A mutation was the only deficient variant identified, supporting findings reported in other tropical Sub-Saharan countries (Beutler 1989b).

The prevalence of the normal wild type G6PD B variant was 59.3 %, of which (36.7 %) were hemizygous males (Gd^B) and (22.6 %) were females (Gd^B/Gd^B). The gene frequency of the wild type Gd^B allele was 0.69 and is comparable to that reported in Senegal (0.68) (Araujo *et al.*, 2006) and Equatorial Guinea (0.72) (Pinto *et al.*, 1996) and Congo (0.57) (Bouanga *et al.*, 1998). Lower frequencies have been reported in Ivory Coast (0.51) (Coulibaly *et al.*, 2000).

When a gene that has some potential for decreasing fitness achieves a high frequency in populations, it can be assumed that it confers a survival advantage in the target population. Equilibrium is said to have been achieved between the advantage and the disadvantage conferred by a gene, and such a gene is said to show balanced polymorphism (Beutler, 1994). The mortality caused by malaria exerted strong selection pressure on a large number of genetic traits and many polymorphisms affecting the RBC seem to have reached high frequencies for this reason (Nagel *et al.*, 1989). These include

the sickle cell trait, a group of genetic disorders caused by sickle hemoglobin (Hb S). In many forms of the disease, the RBCs change shape, upon deoxygenation because of polymerization of the abnormal Hb S. This process damages the RBC membrane, and can cause the cells to adhere to blood vessels. This deprives the downstream tissues of oxygen and causes ischemia and infarction, which may cause organ damage, such as stroke. The disease is chronic and lifelong. Individuals are most often well, but their lives are punctuated by periodic painful attacks. Life expectancy is shortened, but contemporary survival data is lacking. Older studies indicated that sufferers could live to an average of 40 to 50 years, with the average age for males being 42 and the average age for females being 48. Sickle-cell disease occurs more commonly in people (or their descendants) from parts of the world such as sub-Saharan Africa, where malaria is or was common, but it also occurs in people of other ethnicities. As a result, those with sickle cell disease are resistant to malaria since the RBCs are not conducive to the parasites. The mutated allele is recessive, meaning it must be inherited from each parent for the individual to have the disease (Desai & Dhanani, 2004). Thalassemia, another genetic trait, is an inherited autosomal recessive blood disease. In thalassemia, the genetic defect results in reduced rate of synthesis of one of the globin chains that make up Hb. Reduced synthesis of one of the globin chains causes formation of abnormal Hb molecules and this in turn causes the anemia that is a characteristic presenting symptom of the thalassemias. Being a carrier of the disease may confer a degree of protection against malaria, and is quite common among people from Italian or Greek origin, and also in some African and Indian regions. This is probably by making the RBCs more susceptible to the less lethal species *Plasmodium vivax*, simultaneously making the host RBC environment unsuitable

for the merozoites of the lethal strain *P. falciparum*. This is believed to be a selective survival advantage for patients with the various thalassemia traits (Wambua *et al.*, 2006).

The wild type G6PD B variant is present worldwide, but other variants, particularly those resulting in enzyme deficiency, are restricted to specific geographic regions (for example G6PD A and A- in sub-Saharan Africa and G6PD Med in Southern Europe, the middle East, and India), although they may occur at a low frequency in regions where there has been recent gene flow (Vulliamy *et al.*, 1992). The G6PD A- is the only variant thought to provide protection against malarial infection in Africa.

An important question to consider in the presented data is whether the gene pool is in Hardy-Weinberg equilibrium, which was calculated from Hardy-Weinberg equation, The Hardy-Weinberg equilibrium states that allele frequencies in a population remain constant over time, in the absence of forces to change them. Its name derives from Godfrey Hardy, an English mathematician, and Wilhelm Weinberg, a German physician, who independently formulated the equation in the early twentieth century. The statement and the set of assumptions and mathematical tools that accompany it are used by population geneticists to analyze the occurrence of, and reasons for, changes in allele frequency. Evolution in a population is often defined as a change in allele frequency over time. The Hardy-Weinberg equation, therefore, can be used to test whether evolution is occurring in populations (Hartl and Clark, 1997). Using this equation, the expected genotype frequencies can be calculated and then compared to observed frequencies.

The expected genotypic frequencies in the female group were calculated from the Hardy-Weinberg equilibrium equation and obtained 18.0 % for heterozygous and 1% homozygous. In the study, a 15.2 % frequency was observed for the heterozygous females and 2.2 % for homozygous females (Figure 10). A chi-square test was used to determine whether there was significant difference between the expected and the observed genotype frequencies. There was a statistically significant difference leading to the rejection of the null hypothesis stating that the inherited G6PD deficiency alleles are not in Hardy-Weinberg equilibrium. This finding is contrary to a study conducted in Gabon where distribution of the G6PD deficiency alleles was said to be in Hardy-Weinberg equilibrium (Mombo *et al.*, 2003).

From the results of Hardy-Weinberg equilibrium equation, the conclusion is that the G6PD allele frequencies in the population studied are changing in favor of deficient variants due to the selective pressure exerted by malaria. For a disease to exert selective pressure it would have to have a significant effect on morbidity and mortality before reproductive age and to have been exerting these effects for long periods of time. The protection conferred by these innate factors in malaria holoendemic areas is most evident in childhood prior to the development of acquired immunity from repeated *P. falciparum* exposure. G6PD variants are polymorphic and have been amplified in the population because their favorable effects counterbalance the impairment of fitness they may cause. The selective pressure from malaria is very strong, evident from the fact that the G6PD A- variant has risen to high frequencies (2-25%) in Africa (Chan, 2004) despite the potential fatal consequence to the affected individuals. Emerging studies indicate that the

male hemizygotes and female heterozygotes are protected against malaria and this allows an allele linked to the deficiency to eventually reach fixation.

Hemizygous males or homozygous females with more severe enzyme deficiency are prone to hemolytic attack, but this is not equated to better protection from malaria compared to female heterozygotes. Hence, the overall fitness of female heterozygotes is likely to be greater than that of hemizygotes and homozygotes (Ruwende *et al.*, 1995). This situation constitutes a balanced polymorphism and would explain the observed rarity of populations in which frequencies of G6PD deficiency are in excess of 50 % (Luzzatto *et al.*, 1989 and Livingstone, 1985). Hence the magnitude of protection against malaria suggests that a counterbalancing selective disadvantage associated with the deficient genotypes may have prevented G6PD deficiency from reaching high frequencies and eventually achieving Hardy-Weinberg equilibrium.

4.2 CONCLUSION

The findings presented in this study indicate a high prevalence (13.9 %) of the African form of G6PD deficiency (G6PD A-) in children participating in malaria vaccine clinical trial. These findings have important implications in the conduct of malaria intervention studies since: 1) the protection conferred by this trait may confound interpretation of efficacy data as one may ascribe protection to the vaccine yet it is due to presence of G6PD deficiency as a natural protective trait against malaria, 2) inadvertent administration of certain anti-malarial drugs may precipitate hemolytic crises in

individuals with severe form of G6PD deficiency and therefore increase incidences of serious adverse events (SAE).

The conditions described in the previous paragraph raise the question of what would be the appropriate method for G6PD deficiency screening. Though the quantitative method of detecting G6PD enzyme activity is adequate in detecting un-confounded G6PD deficiency, they may fail to identify G6PD deficient individuals recovering from a recent hemolytic crises or female heterozygotes. The results of the study undertaken here indicate that a combination of PCR and RFLP would be appropriate methods for screening G6PD deficiency.

Finally, the G6PD locus in the studied population was not in Hardy-Weinberg equilibrium indicating continuous selection pressure exerted on the locus, probably, by malaria.

4.3 RECOMMENDATIONS

1. Considering the high prevalence of G6PD deficiency observed in this study, it is recommended that screening for G6PD deficiency by genotyping be conducted whenever malaria intervention studies are envisioned in order to identify individuals who may confound interpretation of efficacy data. Equally, such screening will identify individuals who may experience hemolytic attack if treated

with certain anti-malarial drugs. It is also recommended that screening for genetic traits be expanded to include other RBC polymorphisms such as sickle cell traits and hemoglobinopathies that are protective against malaria.

2. Although G6PD is known to confer a certain amount of protection to malaria, the mechanism of this protection is unknown. It is, therefore, recommended that the G6PD deficiency mechanism of action be explored for further elucidation.

3. Since the effect of G6PD deficiency on natural immunity upon vaccination is unknown, it is also recommended that, the influence of the G6PD deficiency on the development of humoral and cell mediated immunity to malaria following vaccination be studied.

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APPENDICES

1. Standard worksheet for PCR analysis of G6PD deficiency mutations

DATE: _ _ / _ _ / 20_ _

Reagent	**Final Concentration**	**25 µL reaction mix**	**×____Samples**
10X PCR Buffer	1X	2.5 µL	µL
25 mM $MgCl_2$	1.5 mM	1.5 µL	µL
10 mM dNTPs	200 µM	0.5 µL	µL
100 µM/ µL Forward Primer	0.4 µM	0.1 µL	µL
100 µM/ µL Reverse Primer	0.4 µM	0.1 µL	µL
5U/µL Taq polymerase	0.75 U	0.15 µL	µL
DNA		2.00 µL	
PCR H_2O	q.s to 25 µL	13.35 µL	µL
TOTAL		= **25.00µL**	= µL

SAMPLES

01._____	11._____	21._____	31_____
02._____	12._____	22._____	32_____
03._____	13._____	23._____	33_____
04._____	14._____	24._____	34_____
05._____	15._____	25._____	35_____
06._____	16._____	26._____	36_____
07._____	17._____	27._____	37_____
08._____	18._____	28._____	38_____
09._____	19._____	29._____	39_____
10._____	20._____	30._____	40_____

CONTROLS

Positive control_____
Negative control No Template Control

CYCLING CONDITIONS

94° C - 10 minutes
94° C - 45 sec.
58° C – 45 sec.
72° C – 45 sec.×30
72 ° C – 7 minutes
04° C - ∞

COMMENTS

_____

PCR DONE BY: _____

1. Standard worksheet for RFLP analysis of G6PD Deficiency Mutations

DATE: _ _ / _ _ / 20_ _

Reagent	Final Concentration	25 µL reaction mix	×____Samples
10X Buffer 4	1X	2.00 µL	µL
4U/µL Enzyme	1U	1.00 µL	µL
PCR Product	q.s to 20 µL	10.00 µL	µL
PCR H_2O		7.00 µL	
TOTAL		**=20.00 µL**	= µL

SAMPLES

01._____	11._____	21._____	31_____
02._____	12._____	22._____	32_____
03._____	13._____	23._____	33_____
04._____	14._____	24._____	34_____
05._____	15._____	25._____	35_____
06._____	16._____	26._____	36_____
07._____	17._____	27._____	37_____
08._____	18._____	28._____	38_____
09._____	19._____	29._____	39_____
10._____	20._____	30._____	40_____

CONTROLS

Positive controls: Normal female (Gd^B/Gd^B) or Male (Gd^B), heterozygous female (Gd^B/Gd^A, Gd^B/Gd^{A-} or Gd^A/Gd^{A-}), homozygous female (Gd^A/Gd^A or Gd^{A-}/Gd^{A-}) or hemizygous male (Gd^A or Gd^{A-}).

Negative control_____

DIGEST CONDITIONS

37°C – 16 hours

COMMENTS

_____

RFLP DONE BY: _____