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Molecular characterization of Glucose-6-Phosphate Dehydrogenase deficiency specific variants among selected populations in malaria endemic areas of Ethiopia.

BY: GETACHEW ABEBE

A Thesis submitted to Addis Ababa University, School of Graduate Studies, Department of Medical Laboratory Sciences in Partial fulfillment of the requirements for the Master of Science Degree in Clinical Laboratory Science (Clinical Chemistry Specialty Track).

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This is to certify that a thesis prepared by **Getachew Abebe Ewonetu** entitled on: "Molecular characterization of Glucose-6-Phosphate Dehydrogenase deficiency specific variants among selected populations in malaria endemic areas of Ethiopia" is submitted in partial fulfillment of the requirements for the Master of Science Degree in Clinical Laboratory Science (Clinical Chemistry Track).

Signed by the Examining Committee:

External examiner	Signature	Date
Internal examiner	Signature	Date
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Chair of Department or Graduate Program Coordinator

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List of Abbreviations/Acronyms

AAU	Addis Ababa University
AHARI	Armauer Hansen Research Institution
bp	base Pairs
CDC	Center for Diseases Control and prevention
DBS	Dried Blood Spot
DIHA	Drug Induced Hemolytic Anemia
DNA	Deoxyribonucleic Acid
EA	Enumeration Areas
EPHI	Ethiopian Public Health Institution
FokI	Gene from Flavobacterium Okeanokoites I
G6PD	Glucose 6-Phosphate Dehydrogenase
G6PDd	Glucose 6-Phosphate Dehydrogenase deficiency
MDGs	Millennium Development Goals
MIS	Malaria Indicator Survey
MboII	Gene from Moraxella bovis II
NADP+	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NIaIII	Gene from Neisseria lactamica III
PCR	Polymerase Chain Reaction
Pf	Plasmodium falciparum
PPP	Pentose Phosphate Pathway
PPS	Probability Proportional to Size
Pv	Plasmodium vivax

RBCs	Red Blood Cells
RFLP	Restricted Fragment Length Polymorphism
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
SNNPR	Southern Nation and Nationalities Peoples' Region
SRS	Simple Random Sampling
sSA	sub-Saharan Africa
TBE	Tri-Borate- Ethylenediaminetetraaceticacid (EDTA) buffer
WHO	World Health Organization

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Operational definitions

Glucose-6-Phosphate Dehydrogenase deficiency: Glucose 6-Phosphate Dehydrogenase encoding gene mutation that can be either of the 3 of variants: G6PD*A, G6PD*A^{-} or G6PD* Mediterranean variant.

G6PD*A variant: G6PDd variant results from a point mutation at 376 nucleotide (376A→G). The mutation can exist at 125bp and 183bp.

G6PD*A^{-} variant: G6PD*A^{-} Variant results from a mutation at 376 nucleotide (376A→G) and 202 nucleotide (202G→A). The mutation can exist at 131bp and 81bp.

G6PD* Mediterranean variant: G6PD* Mediterranean Variant results from a mutation at 563 nucleotide (563C→T). The mutation can exist at 154bp, 98bp and 33bp.

FokI: A restriction endonuclease extracted from an *E. coli* strain that carries gene from *Flavobacterium Okeanokoites* I, and allow to recognizes the A376G mutation (i.e., G76PD*A).

MboII: A restriction endonuclease extracted from an *E. coli* strain that carries gene from Moraxella bovis II, and allow to recognizes the A376G mutation (i.e., G76PD*A^{-} variant).

NlaIII: A restriction endonuclease extracted from an *E. coli* strain that carries gene from Neisseria lactamica III, and allow to recognizes the A376G mutation (i.e., G76PD* Mediterranean variant).

Abstract

Background: Glucose 6-phosphate dehydrogenase deficiency (G6PDd) is an X-linked hereditary genetic defect, affects an estimated 400 million people worldwide. Severe clinical manifestation associated with G6PDd (e.g., chronic hemolytic anemia) depends on the type of G6PD molecular variants and exposure to hemolytic triggers (e.g., antimalarial like Primaquine). However, scarce studies on G6PDd renders the use of Primaquine for effective therapeutic treatment of malaria.

Objective: To determine the availability and characterize selected molecular variants of G6PDd specific genes among selected populations in malaria endemic area of Ethiopia.

Method: A cross sectional study was conducted among selected populations in malaria endemic areas of Ethiopia from July 30, 2014 to January 30, 2015. A total of 523 dried blood spot samples were randomly selected from stored samples of national malaria indicator survey of 2011. Polymerase chain reaction and restricted fragment length polymorphism technique was applied to characterize G6PDd variants as G6PD*A, G6PD*A^- and/or G6PD*Mediterranean. Binary logistic regression was applied to see association ($P<0.05$ is significant) among different parameters.

Result: Of 523 studied dried blood spot samples, 514 (98.28%) had G6PD genotype available, among which G6PDd were detected on 46 (9.0%) samples. G6PD*A (100%) was the only genotype characterized, while neither G6PD*A^- nor G6PD*Mediterranean genotypes were detected. Of all 46 (9.0%) G6PD*A mutation, 25 (4.9%) were male hemizygous, 4 (0.8%) were homozygous females and 17 (3.3%) were heterozygous females. The result also showed G6PDd prevalence variation among regions with 12.06% in Southern Nations Nationalities Peoples, 10.62% in Tigray, 8.51% in Somali, 6.41% in Amhara and 5.26% in Afar. However; there was no statistical significant difference between G6PDd and regions ($P>0.05$).

Conclusion: G6PD*A variant was the only G6PDd genotype detected in this study. G6PD*A variant has almost (90%) the same enzymatic activities with the wild type. Therefore; this result supports the safe use of primaquine, especially the single low dose for transmission interruption of *Plasmodium falciparum gametocyte* and radical cure of *Plasmodium vivax*, as a part of malaria elimination toolkit, among selected populations in malaria endemic areas of Ethiopia.

Key terms: *Glucose 6-phosphate Dehydrogenase deficiency, Polymerase Chain Reaction, Restriction Fragment Length Polymorphism, Malaria, Primaquine, Ethiopia.*

1. Introduction

1.1. Background

Glucose-6-phosphate dehydrogenase deficiency (G6PDd) is the most common and heritable X-chromosome linked enzymatic defect which affects an estimated 400 million people worldwide (1). It was identified in 1956 by Carson and his colleagues (2). This defect is widespread and most frequently seen in Africa, Asia and Mediterranean region (3,4). The cause is mutations in the glucose-6-phosphate dehydrogenase (G6PD) gene, and resulting in protein variants with different levels of enzyme activity associated with wide range of biochemical and clinical phenotypes (3,5).

Even though more than 400 G6PD variants have been identified (6,7), nearly 300 variants have been confirmed by the World Health Organization (WHO) (8,9). More recently cloning and sequencing of the G6PD gene allowed researchers to characterize approximately 186 molecularly distinct variants (10). In sub-Saharan Africa (sSA), three variants occur with polymorphic frequencies greater than 0.1%; G6PD*B (wild type), G6PD*A (A376G) and G6PD*A^- (G202A). Besides G6PD*B which is most common variant in Africa and worldwide, G6PD*A is considered the most prevalent and most studied in sSA (11).

Glucose-6-phosphate dehydrogenase deficiency (G6PDd) has been reported in almost all racial groups with prevalence rates ranging from 0.1% in Japan (12) to 35% in sSA (13). Its global distribution is remarkably higher in malaria-endemic area (10,14). The most common clinical manifestations of G6PDd are neonatal jaundice and acute hemolytic anemia (5,15). Frequently, these patients suffer from cyanosis, headache, fatigue, tachycardia, dyspnea, lethargy, and/or lumbar/sub-sternal pain (3,16). Usually, such clinical manifestations were triggered by an exogenous agent. For instance; primaquine, an anti-malaria drug, has emerged as a major drug trigger of hemolysis in G6PDd individuals (17).

1.1.1. The Genetic basis of G6PD

The glucose-6-phosphate dehydrogenase (G6PD) gene region is one of the first regions of the human genome to be completely sequenced. The total length of the gene is about 18.5 kb and contains 13 exons. Exon 13 is about 800 nucleotides long and contains the translation stop codon. The protein-coding region is divided into 12 segments (18,19).

The gene encoding G6PD is located near the telomeric region of the distal long arm of the X chromosome (band Xq28) (3,7). The G6PD gene's position on the X chromosome has important implications for its population genetics (20,21). G6PD is a sex-linked and very polymorphic gene in populations in which males have only one allele (hemizygous) and females have two G6PD alleles. Thus, females can be either normal or deficient (homozygous), or intermediate (heterozygous) phenotypes, whereas males can be either normal or G6PD-deficient phenotype. The frequency of the deficient phenotype is higher in males than females (21,22).

This Mendelian X-linked gene is one of the most highly polymorphic of the human genome with at least 186 mutations (10). Not all mutations are polymorphic and of public health significance, but many instead appear only sporadically within populations and very rarely associated with the most severe clinical phenotypes (23,24). Most mutations are single point substitutions leading to amino acid substitutions. All known mutations have been found to affect the coding regions of the gene and none described in the regulatory regions, suggesting that reduced enzyme activity levels are associated with enzyme instability, rather than deficiencies in gene expression (10,24).

1.1.2. The G6PD Enzyme structure and function

The G6PD enzyme is a highly polymorphic consists of either dimer or tetramer forms, depending on pH and ionic strength, of a protein subunit made of 514 amino acids. Each subunit binds to a NADP+ molecule for its structural stability (25). The majority of mutations disrupt the enzyme structural stability and thus reduce its overall activity or decreased affinity of G6PD for its substrates (22). The effect of each mutation on enzyme structure and function depends on the location of the substituted amino acid (26,27).

This enzyme is a typical cytoplasmic, a ubiquitously expressed enzyme that has a housekeeping role in all cells, particularly in red blood cells (RBCs) (28). It plays a critical role in maintaining RBCs integrity through catalyzing a key step in the cell's metabolic production of reducing equivalents that maintain redox equilibrium of the cytoplasm. This protects the cell from oxidative attack by radicals derived from oxygen and organic compounds such as drugs and their metabolites (3,5,16). Specifically, it involved in the first step of Pentose Phosphate Pathway (PPP) and plays a pivotal role in the generation of reduced form of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) which in turn crucial to preserve the reduced form of glutathione (figure 1) (22,24,26)

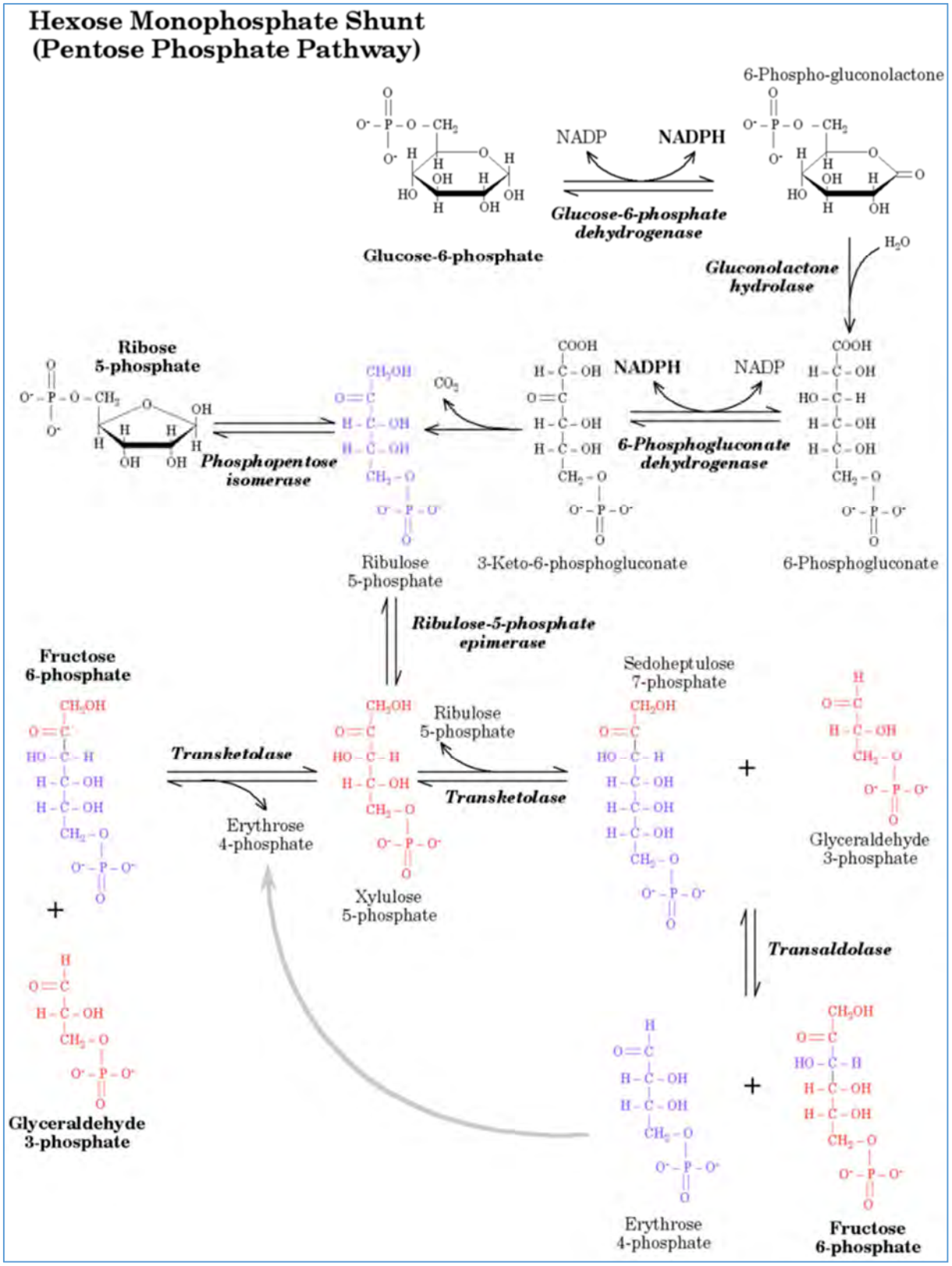

Figure 1: *The chemical reactions catalyzed by G6PD and the Pentose phosphate pathway (PPP)* (29).

1.1.3. Classification of G6PD variants

Enzymatic activity of G6PD varies accordingly as the mutation exists at different level of nucleotide sequence. G6PD*B is the most commonly found enzyme type and it is used as a standard for normal enzyme activity (11). World Health Organization (WHO) classified G6PD variants in to five groups (Class I−V) (7,13). Class I variants have severe deficiency of the enzyme activity and causes chronic nonspherocytic hemolytic anemia (e.g., G6PD*Tokyo). Class II variants have severe deficiency of the enzyme activity without causing chronic nonspherocytic hemolytic anemia. Their enzymatic activities are <10% of normal (e.g., G6PD*Mediterranean). Class III variants have moderate deficiency with enzyme activity 10-60% of normal (e.g., G6PD* A^-**).** Class IV variants have very mild to none deficiency with enzyme activity 60-100% of normal (e.g., G6PD*A). Class V variants have increased enzyme activity (e.g., G6PD*Hektoen) (3,7,13).

1.1.4. The G6PDd and malaria

There is a remarkable geographical distribution similarity between malaria and G6PDd (3). The incidence of G6PDd is high in malaria endemic areas. The explanation for high frequency of G6PDd is due to G6PD deficient variants confer resistance against malaria caused by *Plasmodium falciparum (Pf)* and *Plasmodium vivax* (*Pv*) infections (30). The mechanism conferring protection or resistance in G6PDd subjects may be related to an impaired antioxidant defence in ring-stage parasitized red cells, which could lead to membrane damage, triggering increased removal of infected cells by phagocytosis before parasite maturation to trophozoite and schizonte stages (31). Moreover; during oxidative stress, the loss of potassium from the RBCs and from the parasites can cause the death of the parasites; and hemolysis results from iron that released from the breakdown of hemoglobin by *Plasmodium* parasites, diminished their development rates (26,31).

Glucose-6-phosphte dehydrogenase deficiency (G6PDd) is also thought to be a protection factor against severe manifestations of malaria although studies regarding which individuals, hemizygous males or heterozygous females carriers of deficient alleles, may be protected present discrepancies (31,32). However, in general malaria has been implicated in the spreading of deficient variants in malaria endemic areas. A number of different G6PD deficient variants have reached polymorphic frequencies and each has a characteristic distribution in parts of the world where malaria is currently or was previously endemic (5).

1.1.5. Diagnostic technique for G6PDd

When severe G6PDd complicates malaria infection, treatment with Primaquine, can lead life threatening acute intravascular hemolysis followed by anemia and acute renal failure (33). Hence; to prevent these, malaria patients should be tested for G6PDd before treatment with Primaquine (34). Various tests can be used for the detection of G6PDd, but only a few tests diagnose G6PDd in heterozygous women reliably. Molecular DNA-based methods (genotypic tests) like; polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP), can be used for the diagnosis of heterozygous women but tests are expensive and require sophisticated equipment, which makes unsuitable for large scale study. Molecular diagnoses also allow insight into the severity of the condition for G6PD mutation (variant) types (35,36).

Phenotypic tests, also called biochemical enzyme activity tests, can also be used for diagnosis of all G6PD mutations. However, tests have poor ability to diagnose heterozygotes female deficiency. The most frequently used tests are the fluorescent spot test, cytochemical assay and spectrophotometric assay. The principle is based on the measurement of NADPH production capacity of G6PD. Both fluorescent spot test and spectrophotometric assay based on the formation of fluoresces in which NADPH fluoresces when it is excited with light wavelength of 340nm, whereas cytochemical assay is based on the formation of color in which NADPH converts colorless tetrazolium salt into colored formazan (8,37).

Since its discovery more than 60 years ago, the high prevalence of the defect have made it a favorite tool of clinical chemists/biochemists, epidemiologists, geneticists, and molecular biologists as well as clinicians. So that; this study will try to determine the prevalence and molecular characterization of G6PDd in selected malaria endemic areas of Ethiopia by PCR-RFLP method and provide preliminary data for the concerned bodies targeted for national intervention of malaria elimination in the selected malaria endemic areas of the country.

1.2. Statement of the problem

Glucose-6phosphate dehydrogenase deficiency (G6PDd) is believed to affect about 400 million people globally (1,3,38) or 350 million within malaria endemic countries (39). The striking geographic correlation of G6PDd distribution with the historical endemicity patterns of malaria has led to suggestions that two are linked (3). Even though scarce studies on prevalence and characteristics of molecular variants of G6PDd in Ethiopia, malaria is a serious public health problem in Ethiopia with an average of five million cases a year (40), and the infection is endemic in 75% of the country landmass and putting 68% of the populations at risk of the infection (41).

Different researches showed that the highest prevalence of G6PDd reported in Mediterranean and Africa population including Southern Europe, the Middle East, Southeast Asia, and the central and southern Pacific islands (1). However, due to migration, deficient alleles are now quite prevalent in North and South America and in some Northern European regions (42,43). As a result; G6PDd is the great problem of world wide. Since the defect is X-linked, males and homozygous females inheriting the mutant allele may potentially exhibit signs and symptoms of the disease (44).

Available evidence demonstrates severe G6PDd patients are associated with a variable spectrum of clinical manifestations including: favism, neonatal jaundice, drug and infection induced hemolysis, acute hemolytic anemia, renal failure and mental retardation (45). Apart from these; Cholelithiasis has been detected in more than 38% of subjects with G6PDd (46). In addition; an increased prevalence of proliferative diabetic retinopathy in G6PDd patients with type 1 diabetes, suggesting that G6PDd accelerates the retinal microvascular complications of diabetes (47). In extreme cases, acute hemolysis can lead to permanent neurologic damage or death (20).

To date, molecular characterization of G6PDd variants is not determined in Ethiopia. Despite this, the government of Ethiopia declared to achieve the Millennium development goals (MDGs) goal 6 target 8 by reducing and eradicating malaria among populations (40). At this pivotal time in malaria control, major challenges are relapsing cases of *Pv* and controlling transmission of *Pf* infections (48,49). The only licensed drug active against these kind of parasites is primaquine. However, use of primaquine will result in low to sever hemolysis in G6PDd patients (49,50). Therefore; the aim of this study on determination of G6PDd in context of malaria elimination is to support safe use of primaquine for radical cure and transmission interruption of malaria parasites.

1.3. Significance of the study

It is obvious that the frequency of G6PDd is higher in malaria endemic regions while lower in non-endemic regions, suggesting the relationship between G6PDd and malaria (3). Unfortunately primaquine, the only drug licensed for the radical cure and relapse prevention of *Pv*, and transmission interruption of *Pf*, can trigger severe hemolytic anaemia and even may death in G6PD deficient individuals (48,49). This unique pharmacogenetic association, which is becoming increasingly important as several nations now consider strategies to eliminate malaria transmission rather than control its clinical burden. Therefore; investigations on detection of G6PDd have a vital importance for malaria patients before their treatment with primaquine.

In general; the molecular characterization and determination of G6PDd prevalence in the selected populations in selected malaria endemic areas of Ethiopia helps to generate a base line data to the concerned bodies for developing policies on malaria elimination program specifically on radical cure and relapse prevention of *Pv*, and transmission interruption of *Pf*, through primaquine drug administration. Moreover, it helps to create awareness to the relevant concerned bodies on the importance of screening G6PD deficient individuals before exposing them with oxidative agents other than primaquine to avoid the hemolytic crises among the sufferers.

2. Literature Review

Approximately 186 mutations responsible for G6PDd have been described recently. In sSA, three variants (wild type G6PD* B, G6PD*A and G6PD*A$^-$) were most commonly occurred (9,11,28). A systemic review of literature conducted from selected 2,176 bibliographic sources revealed that G6PD variants showed blatant geographic patterns. The result showed relative homogeneity in the Americas, Africa, and western Asia contrasted sharply with the heterogeneity of variants in China, Southeast Asia and Oceania. From populations of the Americas, Africa, Yemen and Saudi Arabia; G6PD*A$^-$ variant was predominantly searched for and reported. Similarly, G6PD*Mediterranean was predominant in Turkey and Pakistan. However; high variant heterogeneity in the populations of East Asia including China and the Asia-Pacific where no single variant dominated (10).

A representative community based phenotypic G6PDd survey done among 1,734 spatially unique sites predicted a G6PDd allele frequency map across malaria endemic countries. The result showed the highest median prevalence (peaking at 32.5%) predicted across sSA and the Arabian Peninsula; and lowest median prevalence (≤1%) predicted in large malaria endemic areas of America (39).

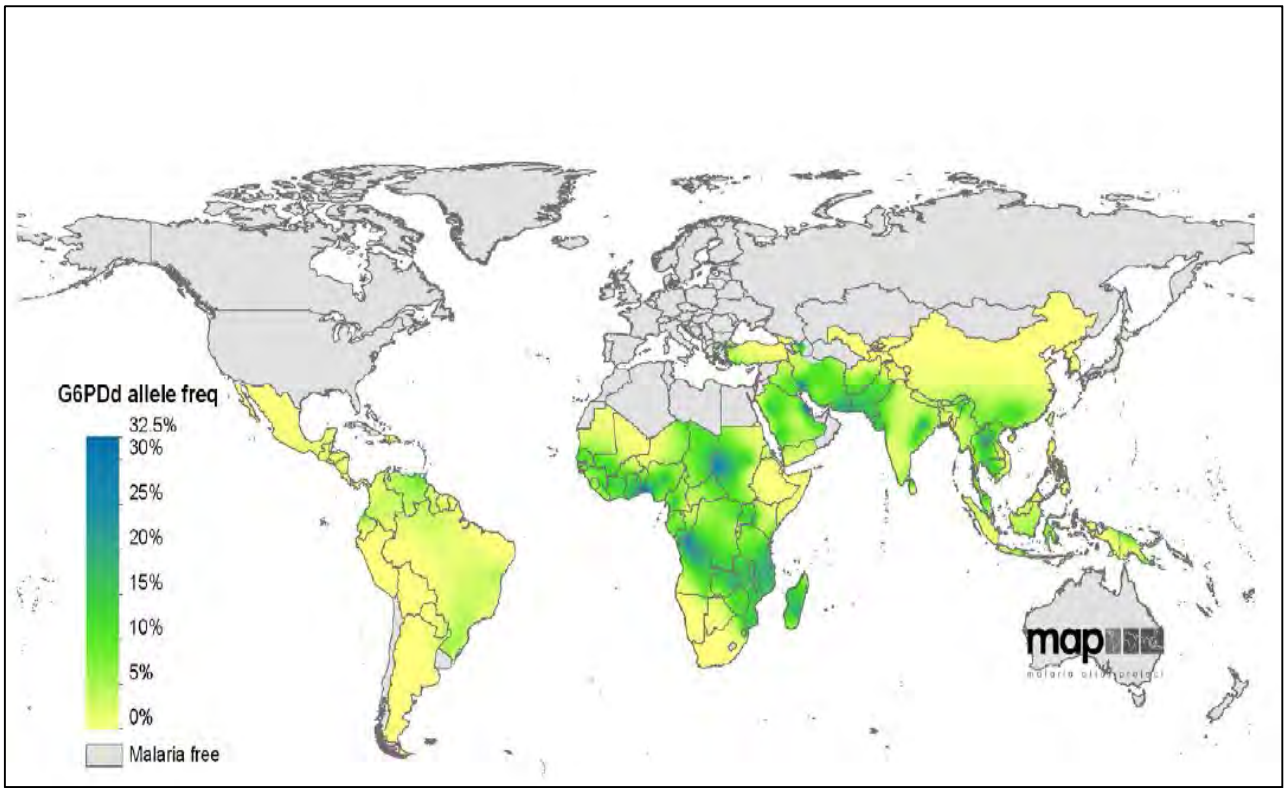

Figure 2: *Map of the global distribution of the median predicted allele frequency of G6PDd (39)*

Most glucose-6-phosphate dehydrogenase (G6PD) deficient individuals are asymptomatic until exposed to hemolytic triggers (oxidative agents) such as infections, certain foods (e.g., fava beans) and drugs (10). In different literatures, drugs were described as hemolytic trigger that causes hemolytic crisis in G6PDd individuals. For instance; dapson, antimalarial (e.g., primaquine), antibiotics (e.g., sulfonamides, quinolones, chloramphenicol and nitrofurantoins) and analgesics (e.g., Phenazopyridine) are some of the drugs that causes hemolysis (7,51). Several clinical disorders, such as diabetes (47) and myocardial infarction (52), and strenuous physical exercise (53) have been also reported to precipitate hemolysis in G6PD deficient sufferers. However, some cases of spontaneous hemolysis have also been reported in patients with severe enzyme deficiency without any exposure to oxidative agents and any other precipitating pathologies (5,54).

Over a third of the world's population lives at risk of malaria (55). In Ethiopia, malaria is the number one health problem with an average of 5 million cases a year (40) and the infection is endemic in 75% of geographical areas and putting 68% population at risk (41). In the context of malaria elimination, therapy must target all infections, including asymptomatic and submicroscopic blood-stage infections, dormant liver-stage hypnozoites as well as clinical cases (49,56). Primaquine has a vital and unique role for this context. However; G6PDd has been detected to cause serious problems in fighting against malaria. Since, primaquine cause severe hemolysis. But, different epidemiological studies reported fear of harmful effects (severe hemolysis) among G6PD deficient patients leads to avoidance of primaquine as a means of malaria transmission-blocking agent (56).

When primaquine is administered to individuals with G6PDd, its metabolites lead to more severe hemolysis. The most probable mechanism is the generation of oxyhemoglobin, which forms hydrogen peroxide. Since G6PD enzyme level is low in G6PD deficient RBCs, these peroxides accumulate and lead to denaturation of hemoglobin. This peroxide can also generate Heinz bodies, which are insoluble aggregates that attach to cell membranes of RBCs (2). Moreover, Primaquine metabolites also lead to GSH depletion and stimulation of the hexose monophosphate. As a result of severe hemolysis, this drug may even cause death in G6PD deficient individuals. Hence, individuals that are required to use antimalarial drugs should be screened very carefully for their tendency to have G6PDd. For effective control and treatment, either a reliable test for detecting G6PDd or an anti-malarial drug that can be safely given to G6PDd individuals is required (28,55).

A cross sectional study conducted in Porto Alegre of Brazil from a sample of 162 females and 186 males peripheral leukocyte that characterize the molecular basis of G6PDd revealed that the G6PDd prevalence was 36 (10.35%). Of 10.35% G6PDd, 6.03% were hemizygous male and 4.32% were female of which 11 (3.17%) were homozygous and 4 (1.15%) were heterozygous. The study characterized the three most common G6PD mutations: G6PD*A, G6PD*A^- and G6PD*Mediterranean. However, G6PD*A^- was the predominant G6PDd variant detected in the area. G6PD*A^- predominance distribution agrees among the ethnic groups that colonized Brazil, especially those of African, Portuguese, Spanish, and Italian origin (57).

Glucose-6-phosphate dehydrogenase deficiency (G6PDd) is a common inherited enzyme defect and an important problem in areas with *Pv* infection because of the risk of hemolysis following administration of primaquine to treat the liver forms of the parasite (56). A genotypic survey carried out from 713 male individuals across nine provinces of Afghanistan in which malaria is endemic showed the prevalence of G6PD*Mediterranean variant (C563T) was 5.6%. The ethnicity distribution have shown that predominance prevalence (8.9%) among Pashtun/Pashai group. The prevalence result also showed significant association on both province and ethnic group (58).

Glucose-6-phosphate dehydrogenase deficiency (G6PDd) is a highly polymorphic enzyme that showed conspicuous geographic patterns (10). A Cross-sectional studies carried out on the staff and students of Advanced Medical and Dental Institute of University Sains Malaysia from 87 venous blood sample, 45 males and 42 females of which 80 Malays, 2 Chinese, 1 Iranian and 4 others. The result revealed that the total prevalence of G6PDd among the subjects was 4.59 %, all of whom were Malay males. The study characterized two G6PD variants: One of the deficient subjects had G6PD Viangchan, while the other three were G6PD Mahidol (59).

The distribution of G6PDd and the molecular genetics of this enzyme vary widely among different ethnic groups (10). A study conducted in Iran from Zanjan and Iranshahr provinces of 1800 unrelated male individuals showed that the prevalence of G6PDd was 2.2% and 19.3% among Zanjan and Iranshahr population, respectively. Despite this, analysis at molecular level shown that the predominant mutation of G6PD in Iran is of Mediterranean type, 72.8% from Zanjan and 85% from Iranshahr populations (60). Another cross sectional study conducted in North West Iranian from 77 G6PDd individuals using PCR-RFLP methods revealed that 62.3 % was a Mediterranean Variants with 77.1% prevalence in men and 22.9% in female (81.8% heterozygote) (61).

A cross sectional study conducted from a total of randomly selected 1810 apparently healthy adult in Baghdad, Iraq showed that the prevalence of G6PDd was 6%. The study used methemoglobin reduction test for screening G6PDd. The PCR/RFLP method for four deficient molecular variants also showed 74.3% Mediterranean (563 C→T), 5.0% Chatham (1003 G→A), 2.0% G6PD*A$^-$ (202 G→A), and G6PD Aures (143 T→C) in none. In addition, the 1311C→T silent mutation was detected in 94.1% G6PD deficient males having the Mediterranean variant (62). However, another study conducted from a total of 580 healthy male Kurdish Iraqis showed that G6PDd prevalence was 10.9% with predominant Mediterranean variant variants (9.48%) and no cases with G6PD*A$^-$ and G6PD Aures were identified (63).

The hemolytic risk of drug among G6PDd individuals depends on a number of factors including drugs and the type of G6PD variant (6,17). A study conducted in Visakhapatnam district of India from 87 cases of malaria with suspected drug induced hemolytic anemia (DIHA) revealed that the prevalence of G6PDd was 35.63% (31 of 87). The result showed that G6PD*Mediterranean variant was the predominant variant in the area with prevalence of 25.29% (22 of 87). Despite this, the result revealed Mediterranean mutation is associated with moderate to severe hemolysis (64).

A genotypic study conducted in clinical trial study participants in six African countries (Burkina Faso, Ghana, Kenya, Nigeria, Tanzania, and Mali) among 2264 microscopically confirmed uncomplicated *Pf* malaria cases, 2045 (90.3%) had G6PD genotype available. Molecular characterization using PCR-RFLP revealed that 22.3% of G6PD*A$^-$ (27.1% in Ghana, 23.6% in Nigeria, 23.2% in Burkina Faso, 23.2% in Kenya, 20.1% in Tanzania and 16.9% in Mali) and 29.4% of G6PD*A (33.6% in Burkina Faso, 30.5% in Ghana, 24.6% in Kenya, 22.7% in Nigeria, 26.9% in Tanzania, and 33.4% in Mali). The study also determined the prevalence between sexes. Of 22.3% of G6PD*A$^-$ genotypes; 7.2% were male hemizygous, 1.8% were females homozygous and 13.3% were heterozygous females. And from 29.4% of G6PD*A genotypes; 13.8% were male hemizygous, 3.7% were homozygous females and 11.8% were heterozygous females (65).

A cross sectional study conducted among 200 asymptomatic malaria cases in a rural community of Burkina Faso showed that the prevalence of G6PDd was 9.5%. G6PDd was significantly higher in men (14.3%) than women (6.0%) and *Pf* asymptomatic parasitaemia was significantly higher among G6PD non-deficient individuals. In addition, the G6PD*A$^-$ was the only deficient variant detected using TaqMan single nucleotide polymorphism assays and PCR-RFLP method (66).

Another cross-sectional done among 194 children of Angola characterize the three common G6PD gene mutations: A376G, G202A and C563T. The result showed that 20.6% A376G (11.3% male hemizygous, 1.0% homozygous female and 8.3% heterozygous females) and 27.9% G202A (10.8% male hemizygous, 5.2% homozygous females and 11.9% heterozygous females). The Mediterranean mutation was not detected among study participants (67).

Even though molecular methods provide more consistent results, screening of G6PDd can also be determined using methemoglobin reduction test and fluorescent spots test. A study carried out from a total of 314 blood donor samples among healthy blood donors in Nigeria showed that the prevalence of G6PDd was 25.5% with highest prevalence shown in the age group 25-34 yrs. ($p<0.05$) (68). With the same determination technique using EDTA whole blood sample, another study was conducted in Sudan among 65 patients with End Stage Renal Failure exposed to different periods of hemodialysis. The result showed that G6PDd prevalence was 60% and the mean of Hemoglobin level, hematocrit, and RBCs count were also lower among G6PDd patients than G6PD non-deficient patients. But Reticulocyte count was higher in G6PDd patients (69).

A cross-sectional epidemiological study of malaria susceptibility conducted during 2006 and 2007 in the Sahel endemic malaria zone of Mali. The study included a total of 712 study subjects recruited from two ethnic groups (375 from Dogon and 337 from Fulani). The study confirmed that the Fulani are less susceptible to malaria, and the G6PD*A^- mutation was rare, less than 1% versus. However, the study reported 7.9% G6PD*A^- mutation from Dogon ethnic group. The study also characterized another G6PD mutation called Betica-Selma (968C/376G). The prevalence was more common in Fulani (6.1%) than Dogon (0.0%) (70).

A longitudinal study done in Uganda assessed G6PD status using PCR-RFLP method. A total of 245 children between 6 months and 9 years were recruited. The result revealed that overall prevalence for the X-linked G6PD*A^- mutation was; 79.59% wild type, 12.65% heterozygous and 7.76% homozygous or hemizygous. There was no statistically significant difference in prevalence and incidence rates of malaria infection among the different G6PD genotypes (71). Another study conducted among 1,437 Gambian children revealed that the prevalence of the G6PD* 202, 968 and 542 mutations was 1.8%, 2.1% and 1.0%, respectively, and higher in boys than girls (72).

A hospital based cross sectional study conducted from 449 febrile patients (210 males and 239 females) attending Gambella hospital of Ethiopia showed that the prevalence of malaria was 266 (59.2%). G6PDd phenotypes was also screened using CareStart™ G6PDd screening test. Overall, 33 (7.3%) individuals were deficient for G6PD enzyme activity. Although the prevalence of G6PDd was slightly higher among males 18 (8.6%) than females 15 (6.3%), the difference was not statistically significant. However, the chance of being G6PDd was significantly higher for the native ethnic groups (Anuak (12%) and Nuer (14.3%)) compared to the 'highlanders'/settlers (73).

3. Objectives

3.1. General objective

- To determine the availability and the selected molecular variants of G6PDd specific genes among selected populations in malaria endemic area of Tigray, Afar, Amhara, Somali, and Southern Nation and Nationalities Peoples' region (SNNPR) of Ethiopia.

3.2. Specific objectives

- To determine the regional prevalence of G6PDd among selected populations in malaria endemic area of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia.
- To determine the association between G6PDd and sexes among selected populations in malaria endemic area of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia.
- To determine G6PD*A molecular variant among selected populations in malaria endemic area of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia.
- To determine G6PD*A^- molecular variant among selected populations in malaria endemic area of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia.
- To determine G6PD*Mediterranean molecular variant among selected populations in malaria endemic area of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia.

4. Method and materials

4.1. Study Area

The study was conducted on selected dried blood spot (DBS) samples collected during the national malaria indicator survey (MIS) of Ethiopia, 2011. This work is limited to samples collected from malaria endemic area of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia.

Ethiopia is the second most populous country in Africa with a population of over 85 million people. Administratively, Ethiopia has nine regional states (Tigray, Afar, Amhara, Oromia, Somali, Benishangul Gumuz, SNNPR, Harrari, and Gambela); and two city administrations (Addis Ababa and Dire Dawa) (74). The major health problems of the country remain largely preventable communicable diseases and nutritional disorders. Malaria is the number one health problem that makes a total of 59,978,887 population at risk. According to the 2006 Ethiopian fiscal year annual report; 55,573 (2.1%) malaria cases in Somali, 63,181 (2.4%) in Afar, 250,911 (9.6%) in Tigray, 708,520 (27%) in SNNPR, and 683,679 (26%) in Amhara region were diagnosed (75). The socioeconomic burden resulting from malaria is immense. Because of this; malaria prevention and control is the major priority program that has enjoyed over the year's utmost government commitment and considerable attention from the health policy makers (40).

4.2. Study design and study period

A cross sectional study was conducted from July 30, 2014 to January 30, 2015.

4.3. Population

4.3.1. Source population

All Households in 252 selected malaria endemic enumeration areas (25 household per enumeration area (EA) were available) of Tigray, Afar, Amhara, Somali, and SNNPR, who participated during MIS of Ethiopia, 2011.

4.3.2. Study population

All persons of all ages in every 4^{th} household and all under five children in every household that were present in randomly selected 25 household in each 252 selected EA of Tigray, Afar, Amhara, Somali, and SNNPR, who were volunteer and gave DBS sample during MIS of Ethiopia, 2011.

4.4. Study variables

4.4.1. Dependent variables

The three G6PDd Variants were the dependent variables. These are;

- ❖ G6PD*A molecular variant
- ❖ G6PD*A- molecular variant
- ❖ G6PD*Mediterranean molecular variant

4.4.2. Independent variables

Demographic data like: age, sex, and address (region) were the independent variables.

4.5. Sample size determination and Sampling

4.5.1. Sample size determination

The required sample size calculated based on single sample size estimation. Therefore; by applying the following formula, the sample size calculated as shown below (76).

$$n = \frac{Z^2\ P\ (1-P)}{d^2}$$

Where; n = sample size, Z = Z statistic for a level of confidence (for the level confidence of 95 percent, Z value is = 1.96), P = expected prevalence or proportion (P = 50 %), and d = degree of freedom (d = 4.5 %). The value of p was taken as 50 % since there was scarce previous related large scale study undertaken. With this assumption, the sample size was 475.

$$n = \frac{1.96^2\ .5\ (1-.5)}{.045^2} = 475$$

But, this thesis was done from stored samples, there might be 10 percent non-response rate. 10 percent of 475 study samples were 48. So that; the sample size was 475 plus 48 which was equal to **523 study samples**. In addition to the expectation of non-response rate, the sample size increase more than what is calculated is mainly due to the study was a large scale community based cross sectional survey and to keep sampling errors as low as possible and to make the sample size more accurate and precise, and representative.

4.5.2. Sampling technique

The first stage of sample selection was based on probability proportional to size (PPS). Based on this, a total of 252 EA were selected. In the second stage, a randomly selected 25 households from the list of potential households in each EA were targeted to collect DBS samples. A total of 12,000 DBS samples collected from all persons of all ages in every fourth household and all under five children in every household that were present in randomly selected 25 household. The third stage of sample selection was based on PPS to recruited 2000 DBS samples for genotypic study. The sampling technique for this study was an extension from recruited 2,000 DBS samples of MIS Ethiopia, 2011. A simple random sampling (SRS) method employed to select proportional study samples from each region based on population at risk size. Based on this; 161 study samples from Amhara, 145 from SNNP, 113 from Tigray, 57 from Afar, and 47 from Somali regions with a total of 523 study DBS samples selected.

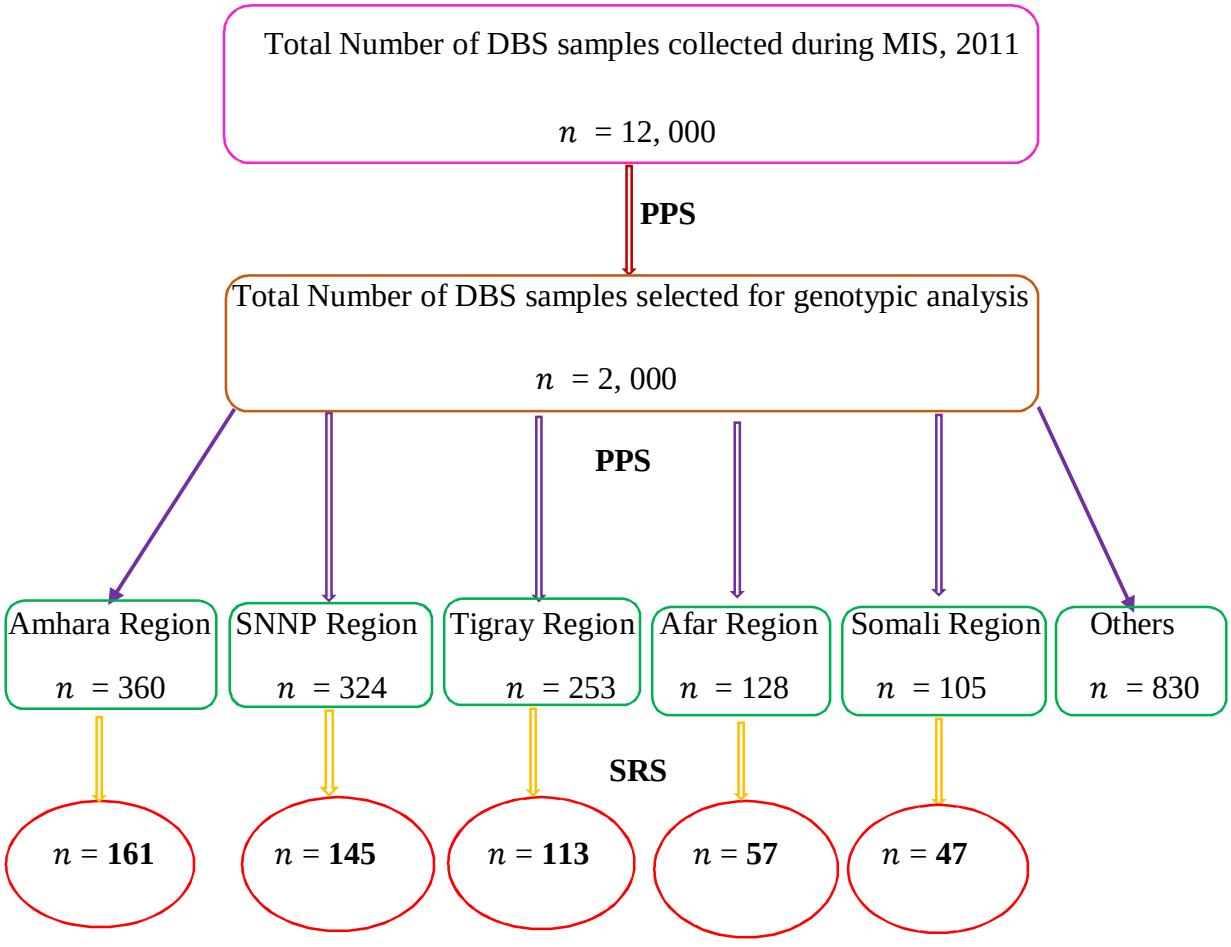

Figure 3. *Schematic presentation of sampling procedure, January 2015.*

4.6. Sample collection and Laboratory test method

4.6.1. Dried Blood Spot (DBS) sample collection

This work is limited to DBS samples collected from selected populations in malaria endemic areas of Ethiopia and used the stored DBS samples and related demographic data from the records of MIS Ethiopia, 2011. However, since 2011 MIS of Ethiopia, DBS samples prepared by taking and applying a small amount of peripheral blood to filter paper cards from finger prick by trained and experienced Laboratory professionals. All DBS samples collected aseptically by applying Universal safety precautions. After proper drying and packing, all blood spots (DBS) shipped to the referral laboratory, EPHI. Finally all samples stored at -20^{o}c refrigerator. Simultaneously with the DBS samples, the demographic data (age, sex, and regions) recorded.

4.6.2. Genomic DNA extraction from DBS sample

The genomic DNA from DBS samples extracted using the QIAamp® 96 DNA Mini Kit. QIAamp DNA Mini kit provide fast and easy methods for purification of total DNA for reliable PCR. This DNA Mini Kit comprises buffer solution like; AL, ATL, Proteinase K, AW1, AW2, and AE buffer. The AL and ATL buffered solution allows to lyse the cell and exposing the genomic material to be extracted. The proteinase K allows to digest the protein and make purified the Genomic DNA from RNA and protein remnants. AW1 and AW2 washing buffer solution are used for purification of the genomic DNA to be extracted. The principle is taking one full spot punched-out from a DBS and placing in 1.5ml micro centrifuge tube and then applying different working reagents based on the protocol with appropriate incubation at different temperature that allows the reaction to be facilitated and allows purified DNA to be extracted. Finally by applying the eluent buffer (nuclease free water) from spin column, it made the Purified DNA available for PCR-RFLP (77).

4.6.3. Genomic DNA amplification by conventional PCR

Conventional PCR used to amplify specific regions of the extracted G6PD gene. The amplification made by using known nucleotide sequence of forward and revers primers. After modification of manufacturer direction, the PCR reaction optimized for each reaction using Promega master mix, 10µm of each primer and Nuclease–free Water. The PCR performed on the DNA Thermal Cycler (MyCycler-BioRad, Hercules, USA) to generate the PCR fragments of the three variants under 1 cycle of 5 min at 94°C, then 32 cycles of 45 sec to 1 min at 94° C, 30 sec to 1 min at 57°C or 64°C, 45 sec to 1 min at 72°C, and final extension at 72°C for 5 min of amplification condition (78).

4.6.4. Restricted Fragment Length Polymorphism (RFLP) technique

Restricted Fragment Length Polymorphism (RFLP) technique employed to characterize the three most common molecular variants of G6PD (G6PD*A, G6PD*A$^-$ and G6PD* Mediterranean). The test principle is all amplified genomic DNA products digested with specific restriction enzymes. This is through mixing of the PCR template (amplified genomic DNA products) with the master mix (specific restriction enzymes, nuclease free water and cutsmart 10x buffer) and incubate the mixture at 37^{o}C for 60 minutes, and inactivating at 65^{o}C for 25 minutes in the PCR machine. The three restriction enzymes used for this technique were FokI for Single Nucleotide Polymorphism (SNP) 376 (G6PD*A variant), NIaIII for SNP 202 (G6PD*A$^-$) and enzyme MboII for SNP 563 (G6PD* Mediterranean) (79).

4.6.5. Genomic DNA separation by agarose gel electrophoresis

Restricted Fragment Length Polymorphism (RFLP) genomic DNA products were run in 2% agarose gel solid support medium for electrophoresis separation. 2% agarose gel solid support medium prepared by mixing 2 grams of agarose powder with 100 milliliters of 1X Tri-Borate-Ethylenediaminetetraaceticacid (TBE) buffer. Melting of prepared solution of the agarose powder made using Microwave oven for 3 minutes. After electrophoresis of RFLP genomic DNA products, the gel photographed, to detect all polymorphic fragments, using Camera installed on UVP Illuminator (80).

Note that the larger fragments fluoresce more intensely. Although each of the fragments of a single class of molecule are present in equimolar proportions, the smaller fragments include less mass of DNA, take up less dye, and therefore fluoresce less intensely.

4.7. Data Interpretation

After electrophoresis of RFLP, the result displayed as a band (cut) form. The interpretation is based on "ladder" set of DNA fragments of known size of 50 base pair (50bp) that can be used to estimate the sizes of the other unknown fragments. For to say there is G6PD*A mutation, it must read a band (cut) at 125bp and 184bp. For to say there is G6PD*A$^-$ mutation, it must read a band at 130bp and 81bp. For to say there is G6PD*Mediterranean mutation, it must read a band at 154bp, 98bp and 184bp (79). It is described and summarized in the table below (Table 1).

Table 1. *Types of G6PD mutations and their related fragment size* (79).

Restriction Enzyme Digestion of PCR products for Mutation Screening				
			Fragment size (bp)	
Mutation	Exon	Restriction enzyme	Normal	Mutated
G6PD*A	5	FokI	308	125, 184
G6PD*A$^-$	3 + 4	NIaIII	211	130, 81
G6PD*Mediterranean	6 + 7	MboII	252	154, 98, 33

4.8. Quality Assurance and Quality control

Standard operating procedures followed strictly. Internal quality control materials for G6PD*A and G6PD*A$^-$ Molecular variants included during running each tests. The tests conducted based on the manufacturers' instruction. In general, the quality assurance principles for pre-analytical, analytical and post-analytical stages applied to assure the quality test result. Those intermediate results repeatedly checked. Visual inspections of neatness of the lab and working bench performed to avoid cross contamination. Properly recording of the daily result was mandatory. In addition, there was a daily follow up by the principal investigator and advisors.

4.8.1. Pre-analytical phase

Since 2011 MIS Ethiopia, the quality of samples assured starting from the time of collection by the responsible body. DBS samples collected aseptically by applying Universal safety precautions. Proper labeling and packing DBS cards in appropriate package assured. After proper drying and packing, all DBS samples shipped to the referral laboratory at EPHI and stored at -20^{o}C refrigerator. However, in this work the pre-analytical phase of quality assurance began on monitoring the stored DBS samples. Monitoring the refrigerator temperature of -20^{o}C made as a daily work. Assembling and sorting all the required material for the work appropriately performed. The neatness of lab and working bench assured before sample analysis began. In addition, to avoid cross contamination with human skin, wearing gloves and lab coat made as a mandatory work.

4.8.2. Analytical phase

Based on the manufacturers' instruction, all DBS samples extracted with in the safety hood. After optimization the PCR machine, all extracted samples analyzed using PCR-RFLP method. The reagents and the test method assessed with a known control materials. The internal quality control materials for G6PD*A and G6PD*A^- variants were available and run in each tests. Calibrated automatic electric balance used for measuring the agarose gel powder. To assure accurate measurement, multiple channel pipetting system done. The standard laboratory procedures also followed and the analysis process was monitored by advisors.

4.8.3. Post-analytical phase

The results recorded in a registration book with the individual's bar-code in daily work. In order to avoid the errors in the results of the test, the reporting was repeatedly checked before. The quality assured results were reported to the advisors.

4.9. Data processing and analysis

The raw data entered in to Microsoft excel and double checked before analysis. Then the data exported to SPSS version 20 for analysis. Percentage and frequency used to show distribution of descriptive data using tables. Figures used to show the RFLP gel electrophoresis result. Bi-variant and multi-variant analysis employed using logistic regression model for further analysis and interpreted based on the odds ratio and level of statistical significant at p-value <0.05.

4.10. Ethical clearance

Ethical issue regarding on confidentiality of study participant made by EPHI at the time of sample collection since MIS Ethiopia, 2011. After brief description of the background and aim of the study, and the possible benefits and risks related to the study participants, consent agreement signed and obtained. Nevertheless, for this work from the initial, the research proposal ethically cleared and approved by the department research and ethical review committee of the department of Medical Laboratory Sciences, School of Allied Health Sciences, Addis Ababa University. Letter of support obtained from the Department of Medical Laboratory Sciences and provided to EPHI. Letter of permission and terms of reference obtained from the project at the EPHI.

5. Results

5.1. Available G6PD Genotype frequency

Of 523 DBS samples selected and extracted using QIAamp® 96 DNA Blood Kit, 516 (98.7%) were appropriately amplified by conventional PCR machine. All 516 amplified products undergoes enzymatic digestion with specific restriction enzymes (i.e., RFLP) and run under gel electrophoreses for separation and detection of specific G6PD mutations. After gel electrophoresis, the result was interpreted appropriately by observing the gel photograph of the visible band form of known fragment size (50 bp ladder). However, 2 (0.4%) of them were not interpreted correctly. Since, the gel electrophoresis result showed that the 2 RFLP products were females, but the 2 samples were registered as male from demographic data sheet. Therefore; from the total of 523 studied DBS samples, G6PD genotypes were available only for 514 (98.3%) samples (Figure 3).

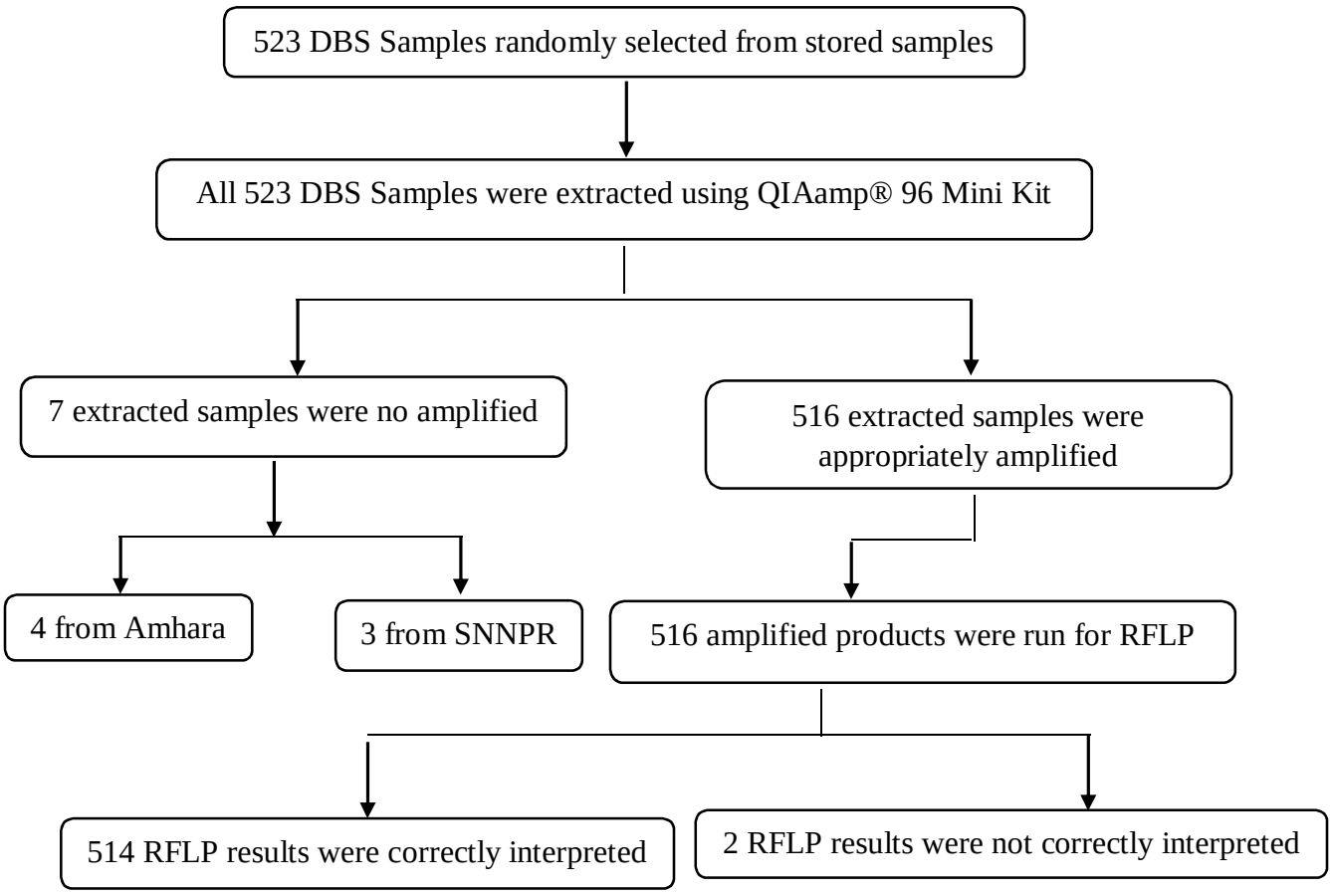

Figure 4. *Genotypic available result After RFLP of 523 studied samples selected from stored DBS samples, collected from selected population in malaria endemic areas of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia, January 2015.*

5.2. Demographic Data

Of 514 genotype available result, 250 (48.6%) were males and 264 (51.4%) were females. The mean age of the study participants was 14.86 with 17.75 standard deviation (age ranges from <1 to 85 years). From a total of 514 studied samples tested for G6PD genotypes; 156 (30.4%) were enrolled from Amhara, 141 (27.4%) from SNNPR, 113 (22.0%) from Tigray, 57 (11.1%) from Afar and 47 (9.1%) from Somali region (Table 2).

Table 2. *Demographic data (age, sex and region) distribution of 514 genotype available samples that were selected from stored DBS samples collected from selected population in malaria endemic areas of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia, January 2015.*

Demographic Variables		**Sex**					
		Male		Female		Total	
		Frequency	%	Frequency	%	Frequency	%
Regions	Tigray	56	10.9	57	11.1	113	22.0
	Afar	33	6.4	24	4.7	57	11.1
	Amhara	76	14.8	80	15.6	156	30.4
	Somali	23	4.5	24	4.7	47	9.1
	SNNP	62	12.1	79	15.4	141	27.4
	Total	250	48.6	264	51.4	514	100
Age group	<5	132	25.7	129	25.1	261	50.8
	5-15	43	8.4	47	9.1	90	17.5
	15-30	28	5.5	42	8.2	70	13.6
	>30	47	9.1	46	9.0	93	18.1
	Total	250	48.6	264	51.4	514	100

5.3. Characterization of G6PDd genotypes and their Prevalence

Of all 514 G6PD genotypes available result, G6PDd detected on 46 (9.0%) samples. Using the PCR-RFLP technique, all studied samples were characterized for the three most common G6PD genotypes; G6PD*A (A376G), G6PD*A$^-$ (G202A) and G6PD* Mediterranean (C563T). Of the three G6PD genotypes, the only G6PD mutation that identified in all studied samples was G6PD*A. Both G6PD*A$^-$ and G6PD*Mediterranean variants were not available in the study site.

The result of G6PD*A genotype was described with respect to sex and regions. Of 46 (9.0%) G6PD*A376G mutations; 25 (4.9%) were male hemizygous, 4 (0.8%) were homozygous females and 17 (3.3%) were heterozygous females. With respect to regions the prevalence of G6PD*A376G mutation was reported: 5.3% (3/57) from Afar, 6.4% (10/156) from Amhara, 8.5% (4/47) from Somali, 10.6% (12/113) from Tigray, and 12.1% (17/141) from SNNPR. The result of G6PDd genotypes with respect to sex and regions described in table below (Table 3).

Table 3. *The prevalence and characteristics of G6PDd genotypes among 514 study subjects; genotypes by PCR-RFLP method from stored DBS samples collected from selected populations in malaria endemic areas of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia, January 2015.*

Region	Sex	*n (%)	G6PD*A376G			G6PD*G202A			G6PD*C563T				*Genotype*
			308	125	184	211	81	130	252	33	98	154	
Tigray	Male	6 (5.3)	-	+	+	+	-	-	+	-	-	-	A
		50 (44.3)	+	-	-	+	-	-	+	-	-	-	B
	Female	1 (0.9)	-	+	+	+	-	-	+	-	-	-	AA
		5 (4.4)	+	+	+	+	-	-	+	-	-	-	AB
		51 (45.1)	+	-	-	+	-	-	+	-	-	-	BB
Afar	Male	2 (3.5)	-	+	+	+	-	-	+	-	-	-	A
		31 (54.4)	+	-	-	+	-	-	+	-	-	-	B
	Female	1 (1.8)	+	+	+	+	-	-	+	-	-	-	AB
		23 (40.4)	+	-	-	+	-	-	+	-	-	-	BB
Amhara	Male	5 (3.2)	-	+	+	+	-	-	+	-	-	-	A
		71 (45.5)	+	-	-	+	-	-	+	-	-	-	B
		1	+	+	+	+	-	-	+	-	-	-	??
	Female	1 (0.6)	-	+	+	+	-	-	+	-	-	-	AA
		4 (2.6)	+	+	+	+	-	-	+	-	-	-	AB
		75 (48.1)	+	-	-	+	-	-	+	-	-	-	BB

Somali	Male	**3 (6.4)**	-	+	+	+	-	-	+	-	-	-	**A**
		20 (42.6)	+	-	-	+	-	-	+	-	-	-	B
	Female	1 (2.1)	+	+	+	+	-	-	+	-	-	-	AB
		23 (48.9)	+	-	-	+	-	-	+	-	-	-	BB
SNNPR	Male	9 (6.4)	-	+	+	+	-	-	+	-	-	-	A
		53 (37.6)	+	-	-	+	-	-	+	-	-	-	B
		1	+	+	+	+	-	-	+	-	-	-	??
	Female	2 (1.4)	-	+	+	+	-	-	+	-	-	-	AA
		6 (4.3)	+	+	+	+	-	-	+	-	-	-	AB
		71 (50.4)	+	-	-	+	-	-	+	-	-	-	BB
Total	Male	25 (4.9)	-	+	+	+	-	-	+	-	-	-	A
		225 (43.8)	+	-	-	+	-	-	+	-	-	-	B
		2	+	+	+	+	-	-	+	-	-	-	??
	Female	4 (0.8)	-	+	+	+	-	-	+	-	-	-	AA
		17 (3.3)	+	+	+	+	-	-	+	-	-	-	AB
		243 (47.3)	+	-	-	+	-	-	+	-	-	-	BB

**n= frequency; %= percent; A=hemizygous male deficiency for G6PD A376G mutation; B=male normal (non-deficient for G6PD A376G mutation); AA = female homozygous deficiency for G6PD A376G mutation; AB = female heterozygous deficiency for G6PD A376G mutation; and BB = female normal (non-deficient for G6PD A376G mutation.*

5.4. Agarose gel (2%) electrophoresis results of the three G6PD genotypes G6PD*A variant (A376G mutation):

After restricted digestion of the amplified products (i.e., RFLP) and gel electrophoresis, the result was displayed in the form of band (cut) which was displayed in the UVP Illuminator adjusted camera after photograph of the gel. In this study, the existence of G6PD*A variant (A376G mutation) was determined after reading of the band at 308bp, 184bp and 125 bp (three bands) in heterozygous state (figure 4-A); and at 184bp and 125 bp (two bands) in hemizygous/homozygous state (figure 4-B). The band reading was made by comparing the fragment/base pair of interest from the known bp ladder (50 bp) that was run along with the unknown bp digested products. The result of G6PD*A genotype (all hemizygous, heterozygous and homozygous stat of deficiency) with positive and negative control was described in the figure below (Figure 5).

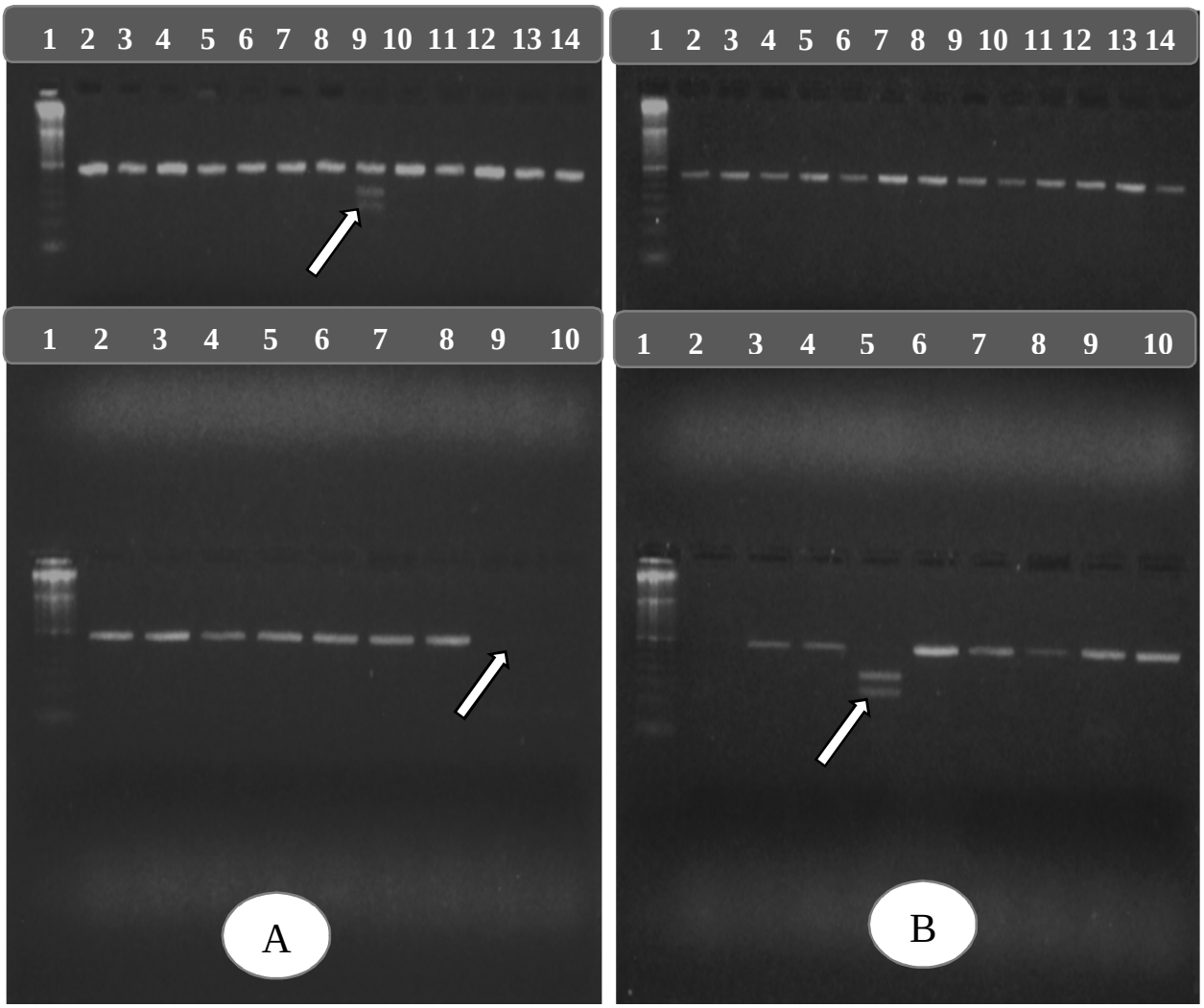

Figure 5. *RFLP and 2% agarose gel analysis of PCR products for G6PD* A376G mutation digested by FokI enzyme, from selected stored DBS samples collected from selected populations in malaria endemic areas of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia, January 2015.*

A. On the top from left to right, lane1: ladder of 50 bp size (DNA marker); lanes 2 to 8: normal samples (non-deficient for G6PD*A); lane 9: the sample has G6PD*A mutation (Heterozygote sample); and lane 10 to 14: normal samples. On the bottom from left to right, lane 1: ladder of 50 bp size (marker); lanes 2 to 8: normal samples; and lanes 9 and 10: negative control.

B. On the top from left to right, lane1: ladder of 50 bp size; lanes 2 to 14 were normal samples. On the bottom from left to right, lane 1: ladder of 50 bp size; lane 2: negative control; lanes 3 and 4: normal samples; lane 5: the sample has G6PD*A mutation (hemizygous/homozygous); and lanes 6 to 10: normal samples.

5.5. The G6PD*A376G mutation and sex

Binary and multiple logistic regression analysis was carried out to investigate whether G6PD*A (A376G mutation) variant and its form might be associated with sex. The analysis revealed that there was no statistical significant association between G6PD*A376G mutation and sex (*P>0.05*) (Table 4). But, hemizygous/homozygous form of G6PD*A376G mutation and sex had statistical significant association (*P<0.05*) (Table 5).

Table 4*. Association between G6PD*A376G mutations and sex among 514 studied samples by PCR-RFLP technique from stored DBS samples collected from selected populations in malaria endemic areas of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia, January 2015.*

Sex	G6PD*A variant		OR (95% CI)	*P-value*
	Deficient	Normal		
Male	25 (4.9%)	225 (43.8)	1.286 (0.700, 2.361)	0.418
Female	21 (4.1%)	243 (47.2)	1	

Table 5*. Association between hemizygous/homozygous form of G6PD*A376G mutation and sex among studied samples selected from stored DBS samples collected from selected populations in malaria endemic areas of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia, January 2015.*

			OR (95%CI)	***P-value**
Crude for hemizygous/homozygous form of G6PD*A376G mutation and sex		Male	**7.222** (2.476, 21.065)	**0.001**
		Female	1	
Regions	Tigray		0.722 (0.263, 1.982)	0.527
	Afar		0.361(0.075, 1.731)	0.203
	Amhara		0.421 (0.147, 1.204)	0.107
	Somali		0.826 (0.207, 3.303)	0.787
	SNNPR		1	
Age group	+5		0.563 (0.207, 1.531)	0.261
	5-15		1.013 (0.327, 3.143)	0.982
	15-30		0.629 (0.147, 2.691)	0.532
	+30		1	

**Hemizygous = male deficiency (X^dY), Homozygous = female two X-Chromosome deficiency (X^dX^d), and *P-value <0.05 = statistical significant association between variables.*

5.6. The G6PD*A376G mutation, regions and age group

This study result showed that the highest prevalence of G6PD*A376G mutation (12.06%) was seen in SNNPR, but binary logistic regression analysis revealed there was no statistical significant difference of G6PDd prevalence in regions (*P>0.05*). Similar to Regions, the binary logistic regression analysis also revealed there was no statistical significant difference of G6PD*A376G mutation frequency among age groups (*P>0.05*). The table below summarizes the regression analysis between G6PD*A376G mutation and regions, and age groups (Table 6).

Table 6. *Association between G6PD*A376G mutation and regions, and age groups among 514 studied samples selected from stored DBS samples, collected from selected populations in malaria endemic areas of Tigray, Afar, Amhara, Somali, and SNNPR of Ethiopia, January 2015.*

		G6PD*A Variant			
		Deficient	Normal	**OR (95% CI)**	***P-value***
Regions	Tigray	12 (10.6%)	101 (89.4%)	1	
	Afar	3 (5.3%)	54 (94.7%)	0.468 (0.126, 1.729)	0.255
	Amhara	10 (6.4%)	146 (93.6%)	0.576 (0.240, 1.385)	0.218
	Somali	4 (8.5%)	43 (91.5%)	0.783 (0.239, 2.565)	0.686
	SNNPR	17 (12.1%)	124 (87.9%)	1.154 (0.527, 2.528)	0.721
Age groups	+5	22 (8.4%)	239 (91.6%)	1	
	5-15	11 (12.2%)	79 (87.8%)	1.513 (0.702, 3.258)	0.290
	15-30	5 (7.1%)	65 (92.9%)	0.836 (0.305, 2.292)	0.727
	+30	8 (8.6%)	85 (91.4%)	1.022 (0.439, 2.383)	0.959

**OR = Odd ratio. 95% CI = 95% Confidence interval, and P>0.05= no statistical significant difference between variables.*

6. Discussion

G6PDd is a common inherited hematological disorder. Acute hemolytic anemia is one of the most common clinical manifestations associated with G6PDd. The severity of hemolysis varied depending on the types of G6PD molecular variants and/or triggering factors (6,17). Among the molecular variants; G6PD*A$^-$ causes mild hemolysis, whereas G6PD*A have increased enzyme activity and doesn't cause hemolysis in the low dose of triggering factors (23). Several phenotypic and genotypic studies were done in African populations to determine the prevalence and molecular variants of G6PDd (66). Genotypic studies allows to characterize specific G6PD molecular variants and helps to estimate the population at risk for hemolysis. While in Ethiopian no genotypic studies were done, nevertheless phenotypic study was done to detect G6PDd prevalence (73).

Hemolysis associated with G6PDd has long since been described as a serious adverse event for a number of antimalarial (17). Primaquine, the only licenseddrug for radical cure of *Pv* and promising drug effective against *Pf* sexual stages, induces a dose-dependent hemolysis among G6PDd individuals. The use of primaquine in malaria endemic areas should be balanced by the potential risk of hemolysis in G6PDd individuals hence the need for accurate estimates of G6PDd prevalence and characterize the specific molecular variants (33,56). As a result; the current study determined the overall prevalence of G6PDd and detected availability of G6PD*A, G6PD*A$^-$ and G6PD*Mediterranean molecular variants using PCR-RFLP method.

The present study result shows the overall prevalence of G6PDd is 9.0%. The prevalence in the current study is in consistent with previous reports from Burkina Faso (9.5%) (66), Kurdish Iraq (10.9%) (63), and Brazil (10.35%) (57). However, it is slightly higher than the prevalence reported from the previous phenotypic study (CareStart™ G6PDd screening test) done in southwest Ethiopia (7.3%) (73). These variations could be explained not only by the larger sample size used in the current study (514 Vs 449), but also by the diagnostic methods used for the detection of the G6PDd (PCR-RFLP technique). Since, G6PD phenotyping has its own limitation on identifying heterozygous female conclusively as deficient (i.e. poor specificity) (37). In addition, this variation could be due to geographical variation and molecular heterogeneity of G6PD mutations among the study subjects. Since, G6PDd is a highly polymorphic enzyme shows blatant geographic patterns and shows molecular heterogeneity among different ethnic groups (10).

In addition, the current study also try to compare the overall prevalence of G6PDd to other studies done in Africa and other world. The prevalence is slightly higher than the previous reports from Malaysia (4.59%) (59) and Baghdad (6%) (62). The higher result in this study could be variation of the study groups (healthy adults in Baghdad), and Molecular heterogeneity, i.e., the difference in targeted genotypes detected (G6PD*Mahidol and G6PD*Viangchan in Malaysia). Because, this study detected G6PD* A376G genotypes as a predominance G6PD molecular variant. However,the prevalence in this stud is lower than the prevalence of previous reports from six African countries (22.3%) (65), Nigeria (25.5%) (68) and India (35.63%) (64). It is not surprising that the current finding is divergent from those reported for particular malaria patients (malaria patients in six African countries and DIHA suspected malaria patients in India). Furthermore, these differences could be due to more of deficient subjects were in age group 25 to 34 in Nigeria ($p<0.05$) (68) and predominant genotype detected was G6PD*Mediterranean in India (64).

Molecular analyses of G6PD variants among Africans revealed a diversity of G6PD mutation (wild type G6PD* B, G6PD*A and G6PD*A- were most common) with G6PD*A- predominance (10). Similarly; despite G6PD*A- predominance among Americas and West Asia (Yemen and Saudi Arabia), G6PD*Mediterranean was predominant in some West Asian countries (from Turkey to Pakistan) (39). However, the present study revealed G6PD* A mutation was the predominant genotype (100%) in which the prevalence was 9.0%. It is lower than the reports from six African countries in which the prevalence of G6PD*A variant was 29.4% (33.6% in Burkina Faso, 30.5% in Ghana, 24.6% in Kenya, 22.7% in Nigeria, 26.9% in Tanzania, and 33.4% in Mali) (65). G6PD*A mutation has 90% of the enzyme activity of the wild type (G6PD*B) (16) and hence use of oxidative agents including antimalarial drugs such as primaquine at low dose for therapeutic purpose, doesn't harmG6PDd sufferers to hemolytic crises (i.e., oxidative stress).

In Ethiopia, malaria is the number one health problem with an average of 5 million cases a year (40) and the disease is endemic in 75% of the country, putting 68%Population at risk (41). In the context of malaria elimination, primaquine has a vital and unique role for treating both relapsing and resistant malaria. However, the fear of primaquine toxicity (i.e. hemolysis) problem with G6PDd hampered the use of the drug as malaria elimination toolkit (17,33). An understanding of the therapeutic risks from primaquine in relation to the G6PD variants predominant in each region (100% G6PD*A variant in this study) will contribute to the body of evidence on which to base

policy. Therefore; this study result (Both G6PD*G202A and G6PD*C563T mutation that can cause mild to severe hemolysis, is not detected) supports the safe use of primaquine especially the single low dose for transmission interruption of *Pf* malaria in selected malarial endemic areas of Ethiopia. Consequently, the present finding supports the country policy makers to formulate the policy relating to malaria elimination for selected malaria endemic regions.

The G6PD* A^- variant (G202A mutation) is the most common G6PDd variant in Africa, with a frequency that ranges from 0–25% (9). While the present study characterized the two most common sub-Saharan Africa G6PD mutations (G6PD*A and G6PD*A^-) using PCR-RFLP technique, G6PD* A^- mutation is not detected (0% frequency) from study participants. This finding is in contrast to an earlier reports from Uganda (20.41%) (71), Burkina Faso (9.5%) (66) and Mali (7.9%) (70). Absence of G6PD*A^- variant in the current study area is most likely due to geographical variation. In addition, this variation proved that the molecular heterogeneity G6PDd which had seen among different populations (10). This makes the study population in selected malaria endemic area being more advantageous from not to be at risk of hemolysis resulted from G6PD*A^- (G202A mutation).

The current study also determined G6PD*Mediterranean variant (C563T mutation), nevertheless it revealed 0% prevalence. This finding is in agreement with the study done in Angola in the Mediterranean mutation was not detected (67). However, this finding is in contrast the previous reports from Afghanistan frequency of 5.6% (58), Iraq of 74.3% (62), Iran of 62.3 % (61) and India of 25.29% (64). G6PD*Mediterranean variant is more dominant/prevalent in many Middle Eastern countries, Indian subcontinent and has been documented as far east as China, Malaysia and Singapore (39). Therefore; this study confirmed that there is different ancestry of the population and absence of genetic crossovers from Mediterranean regions. Hence, type of G6PDd variant are even country specific and varies among different populations (10).

The prevalence of G6PDd was higher among males (54.35%) than females (45.65%) in this study. However, there was no statistically significant difference of G6PDd between sexes. The current finding is in agreement with the previous phenotypic study done in Ethiopia where no significant difference of G6PDd frequency between sexes ($P>0.05$) (73). However, the current study contradicted from the genotypic study done in Burkina Faso (14.3% males and 6% females) where the G6PDd prevalence was significantly higher in men compared to women ($P<0.05$) (66).

Moreover, this study also reported the G6PDd prevalence of females in terms of heterozygous and homozygous states. Of 45.65% deficient females, 36.96% were heterozygous and 8.69% were homozygous state. However, a genotypic study done in Burkina Faso reported 6% homozygous and 27.6% heterozygous females (66). Despite this, the study reported the heterozygous females as normal (non-deficient). Therefore, in the current study the significant variation of G6PDd between sexesis not seen probably due to inclusion of heterozygous female participants from G6PDd report as deficient.Since, the deficient status of heterozygous females depend on the ration of the ratio of normal to deficient RBCs (the phenomenon of Lyonization) (28).

Due to X- linked nature of this enzymatic defect, deficient phenotype is higher in males owing to males being hemizygous in which one allele expresses the deficient phenotype (1,21,22). The present study proves that there is statistical significant difference of G6PDd form (hemizygous/homozygous form) in between sexes. This means; once the individuals are deficient for G6PD, the hemolytic risk of G6PDd is higher in male than female. Because males have only one G6PD allele and females have two G6PD alleles and thus females can have a chance/probability of being intermediate for deficiency, they can be normal or intermediate (heterozygous state). The present study also assessed the relation between regions and G6PDd. But, the study revealed there was no statistical significant difference them. However, different studies showed the existence of G6PD variation among different geographical locations and ethnic groups (4,10). The present study is also not shown the G6PDd variation among ethnic groups.

7. Conclusion and Recommendation

This study identified only the availability of G6PD*A molecular variants (G6PD*A376G) mutations in the study site. In addition to that the study revealed that there was no statistical significant association between G6PD*A376G and the demographic variables like; Sex, Age group and Regions. As it showed in different studies both G6PD*G202A and G6PD*C563T mutations, that were not detected in this study, causes moderate to severe hemolysis even without exposure to oxidative agents. However, G6PD*A376G mutation that was detected in this study G6PD*A, doesn't cause hemolytic crises even with the presence of exposure to low dose of oxidative agents (hemolytic triggers); since, G6PD*A376G mutations has almost (90%) the same enzymatic activities with the wild type. Therefore; this finding supports the safe use of those oxidative agents that have therapeutic effects especially antimalarial agents (e.g., Primaquine) at the single low dose for transmission interruption of *Pf* gametocyte and radial cure of *Pv*, as a part of malaria elimination toolkit in selected malaria endemic areas of Ethiopia. But further study targeted on other variants that can cause severe hemolysis should be conducted to make sure that safe use of those oxidative agents like; drugs, chemicals and others, which have important values (therapeutic effect) for managing and treating G6PD deficient individuals.

8. Limitation of the study

Lack of data relating to hemoglobin level, status of malaria, and ethnicity from the records were the limitation of the study. Due to this, this study couldn't show whether there is statistical significant association between G6PDd and hemoglobin level, malaria status and ethnicity. Moreover, lack of positive internal quality control material for G6PDd*C563T variant (G6PD*C563T mutation) was the other limitation of the current study.

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ANNEXES

Annex I

1. Protocol for DNA Purification from DBS (QIAamp DNA Mini Kit)

1.1. Purpose

- This protocol provides a manual method for extracting DNA from DBS on filter paper when used in conjunction with Qiagen QIAamp®96 DNA Mini Kit.

1.2. Materials and equipment

- QiagenQIAamp®96 DNA mini Kit which contain
  - ✓ ATL, AL, Proteinase K, AW1, and AW2 washing buffer solution
  - ✓ Spine column tube and 2ml collection tube
- Ethanol (96 – 100%)
- DBS puncher (for cutting strips of filter paper with DBS)
- 70% Ethanol
- Incubator at different temperature (85^{O}C, 56^{O}C, and 70 ^{O}C)
- Centrifuge, Test tube rack, 1.5ml microcentrifuge tube
- Calibrated Micropipets (20 µL, 50 µL, 500 µL & 1000 µL)
- Refrigerator (-20°C)

1.3. Safety and Precautions

- Use proper lab etiquette when working in the laboratory. Wear gloves, lab coat and safety glasses when handling specimens.
- Use appropriate safety precautions when using DBS puncher. Wipe the cutting tool with 70% alcohol between samples.
- Ensure that all equipment (pipettes, centrifuge, incubator, etc.) is properly calibrated prior to use.
- Take utmost care during pipetting steps to prevent cross contamination of samples.
- Ensure that Buffer AW1 and Buffer AW2 have been prepared
- Store extracted DNA at -20 °C
- Check the bottles of ATL and AL buffers. If a precipitate has formed, dissolve by incubating at 56 °C before use.
- All centrifugation steps are carried out at room temperature (15–25°C).

1.4. Procedures

1. Place 3 punched-out circles from a DBS into a 1.5 ml microcentrifuge tube and add 180 µl of Buffer ATL.
    - ❖ Cut 3 mm diameter punches from a DBS with a single-hole paper puncher.
2. Incubate at 85°C for 10 min. briefly centrifuge to remove drops from inside the lid.
3. Add 20 µl proteinase K stock solution. Mix by vortexing, and incubate at 56°C for 1hr. briefly centrifuge to remove drops from inside the lid.
    - ❖ Note: the addition of proteinase K is essential.
4. Add 200 µl Buffer AL to the sample. Mix thoroughly by vortexing, and incubate at 70°C for 10 min. briefly centrifuge to remove drops from inside the lid. To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed immediately and thoroughly.
    - ❖ Note: do not add proteinase K directly to Buffer AL. A white precipitate may form when Buffer AL is added to the sample. In most cases, the precipitate will dissolve during incubation. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.
5. Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove drops from inside the lid.
    - ❖ It is essential that the sample and ethanol are mixed thoroughly.
6. Carefully apply the mixture from step 5 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. Close each QIAamp Mini spin column to avoid aerosol formation during centrifugation.
7. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.

9. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
    - ❖ This step helps to eliminate the chance of possible Buffer AW2 carryover.
10. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 150 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.
11. Storing the extracts at -20^{O}C.

1.5. References

- ❖ Handbook for Qiagen QIAamp®96 DNA Mini kit (77)

2. Protocol for Genomic DNA Amplification using Promega master mix

2.1. Purpose/description

- ❖ PCR Master Mix has been optimized for use in routine PCR reactions for amplifying DNA template in the range of 0.2–2kb.

2.2. Product Components

- ❖ PCR Master Mix includes Nuclease-Free Water and PCR Master Mix, 2X.
- ❖ PCR Master Mix, 2X contains: 50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM $MgCl_2$.

2.3. Storage Conditions

- ❖ Minimize the number of freezethaw cycles by storing in working aliquots. Product may be stored at 4°C for up to three months. Mix well prior to use

2.4. Safety and Precautions

- ❖ Due care should be exercised with all Promega products to prevent direct human contact. In no event shall Promega be liable for claims for any other damages.

2.5. Procedures

1. Thaw the PCR Master Mix at room temperature. Vortex the Master Mix and then spin it briefly in a microcentrifuge to collect the material in the bottom of the tube.
2. Prepare one of the following reaction mixes on ice:
   - ❖ Preparation of PCR working solution for SNP 376 & 202

PCR for SNP 376, 202 and 563	1 x (25µl)	100x (1700 µl)
Promega master mix (Taq in)	12.5 µl	1250 µl
Primer 376/202/563 Forward, 10µm	0.25 µl	25 µl
Primer 376/202/563 Reverse, 10µm	0.25 µl	25 µl
Nuclease –free Water	4 µl	400µl
DNA template	8 µl	

Note: DNA amount based on Ethiopia 2011 survey samples: and

DNA volume increased to have better bands

3. Placing each reaction mixes containing PCR wells in to conventional PCR machine
4. Set the PCR machine reaction conditions appropriately (guidelines for Amplification)

PCR reaction conditions (**SNP 376 and SNP 202**)	Initial denaturation	94°C	5 min	1 cycle
	Denaturation	94°C	45 sec	32 cycles
	Annealing	64°C	30 sec	
	Extension	72°C	45 sec	
	Final extension	72°C	5 min	1 cycle
	Hold	4°C	∞	
PCR reaction conditions (**For SNP 563**)	Initial denaturation	94°C	5 min	1 cycle
	Denaturation	94°C	1 min	32 cycles
	Annealing	57°C	1 min	
	Extension	72°C	1 min	
	Final extension	72°C	5 min	1 cycle
	Hold	4°C	∞	

2.6. Reference

- ❖ Promega master mix usage information. Accessed at www.promega.com (78).

3. Protocol for RFLP Technique

3.1. Purpose/description

- This technique allows to characterize the three G6PD molecular variants based on usage of specific restriction enzymes, which restricts/cuts at specific base pair.

3.2. Product components

- 1X Cutsmart buffer:
  - ✓ 50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate AND 100 µg/ml BSA
- FokI, NIaIII and MboII restriction endonuclease enzymes
- 50bp orange color DNA ladder comes supplied with 1 vial of gel loading dye.

3.3. Product Source

- FokI: An E. coli strain that carries the FokI gene from Flavobacterium okeanokoites
- NIaIII: an E. coli strain that carries the NlaIII gene from Neisseria lactamica
- MboII: an E. coli strain that carries the cloned MboII gene from Moraxella bovis

3.4. Important notes

- CutSmart Buffer:
  - ✓ Making it significantly easier to set up the double digest reactions.
- FokI enzyme:
  - ✓ Can cleave between virtually any two nucleotides by constructing a complementary oligonucleotide to the sequence to be cleaved.
  - ✓ Overdigestions of >5 units of FokI per µg of DNA and incubation times >2 hours are not recommended.
  - ✓ Star activity may result from a glycerol concentration of >5%
- NIaIII:
  - ✓ Cleaves to leave a 3´ CATG extension which can be ligated to DNA fragments generated by SphI
  - ✓ NaCI and KCI inhibit activity, $(NH4)_2\ SO_4$ does not.
  - ✓ The half life of NlaIII is 6 months at -20°C.
  - ✓ For long term storage, store at -70°C.
- MboII enzyme:

- ✓ MboII produces DNA fragments that have a single-base 3´ extension which are more difficult to ligate than blunt-ended fragments.
- ✓ Incubations longer than 1 hour are not recommended.
- ✓ May exhibit Star Activity in 1.1 Buffer.
- ✓ Requires two copies of its recognition sequence for cleavage to occur

3.5. Storage Temperature

- ❖ CutSmart® Buffer and Restriction enzymes (FokI, NIaIII and MboII)
  - ✓ @ -20°C
- ❖ 50bp orange color DNA ladder and gel loading dye
  - ✓ At room temperature

3.6. Heat inactivation

- ❖ FokI, NIaIII and MboII: inactivated at 65°C for 20 minutes

3.7. Safety and Precautions

- ❖ Avoid direct contact of restriction enzyme with human skin and other body parts
  - ✓ May cause irritation to skin, eyes, and respiratory tract, may affect kidneys
- ❖ Unless, they are not a dangerous substance or mixture according to the Globally Harmonized System.

3.8. Procedures

1. Thaw the CutSmart and restricted enzymes at room temperature.
2. Prepare one of the following reaction mixes on ice:

Master mix volume		10 µl
	Nuclease free water	7.5 µl
	Cutsmart 10x buffer	2 µl
	FokI/ NIaIII/ MboII	0.5 µl
PCR product (template) volume		10 µl
Total reaction volume		20 l

3. Vortex the reaction mixes and incubate at 37ºC for 60 minutes and inactivating at 65ºC for 25 minutes in PCR-machine after adjustment the reaction conditions.

3.9. Reference

- ❖ Cutsmart buffer, FokI, NIaIII and MboII restriction enzymes usage information. Accessed at www.neb.com/products (79).

4. Agarose gel solid medium (2%) preparation procedures

1. Measuring 2 grams of agarose powder using electronic digital balance
2. Measuring 100 milliliter of 1X Tri-borate- Ethylene Diamine Tetra Acetic acid (EDTA) buffer (TBE buffer) using graduated cylinder.
3. Mixing and stirring the Agarose powder with the TBE buffer
4. Heating the mixed solution in Microwave oven until completely dissolved (usually three minutes).
5. Adding 10 milliliters of nucleic acid stain to the melted solution (gel) for nucleic acid visualization
6. Cooling the mixture to 60^{o}C and pouring into the casting tray for solidification.
7. Placing the comb in its appropriate position
8. Immediately removing the comb after the gel is solidified
9. Keeping the gel in its plastic and pouring the TBE buffer until to fully immerse the gel during electrophoresis
10. After mixing 5 µl PCR product with 2 µl loading dye, loading the mixed products in the wells.
11. As nucleic acids are negatively charged, wells should be placed towards the negative electrode.
12. Connecting the electrode with power source and adjusting the power at 80 volts.
13. Final running 2% gel and check the gel on UVP and record the band in comparison with the ladder of known molecular weight.

❖ Reference: Principles and Practice of Agarose Gel Electrophoresis. Accessed at https://www.wou.edu/ las/physci/ch462/Gel%20Electrophoresis.pdf (80).