SCHOOL OF GRADUATE STUDIES, COLLEGE OF NATURAL SCIENCES
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(APPLIED GENETICS STREAM)

Study of the Allelic Variants of Glucose-6-phosphate Dehydrogenase (G6PD) Using PCR-RFLP in the Oromia, Gambella and Benishangul Gumuz Regions of Ethiopia.

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ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PD) deficiency has been considered as the most commoninherited enzymopathic disorder of the red blood cells, which affects more than 400 million people worldwide. Since the G6PD gene in human is X-linked gene more males are affected than females. G6PD deficiency is particularly prevalent in historically malaria-endemic areas. The main objective of this study is to study the allelic variants of Glucose -6- phosphate dehydrogenase (G6PD) locus prevalent in malaria endemic areas of Oromia, Gambella and Benishangul Gumuz regions in Ethiopia. In this study, blood sample of 620 individuals (326 females and 294 males) were collected from malaria endemic Oromia, Gambella and Benishangul Gumuz regions and stored at -20°C till used. Negative and positive samples were selected as the non-G6PD deficient and deficient control groups was tested for the African A type 376 G →A, African A- A376G & G 202A and 563C→T mutations. Molecular characterization of 620 individuals characterized to informative SNPs using PCR-RFLP techniques revealed an overall prevalence of G6PD Africa A (376 A→G) 10% with 0.0652 from Oromia, 10.5% with 0.074 from Gambella and 9.6% with 0.063 allele frequency from Benishangul Gumuz malaria endemic areas. The G6PD Africa A (376 A→G) was the most common, but African A- and Mediterranean type variants were not observed in this study. The G6PD variant identified in this study can contribute to the evidence-based use of antimalarial drugs like primaquine (for P. *falciparum* and P. *vivax)* treatment strategies that could greatly accelerate the elimination of malaria transmission in the study area. Population-based genotyping studies could be important to identify the distribution of dominant variants in the study regions.

Keywords: Characterization, A376G/ G 202A, G6PD, PCR-RFLP, Prevalence Oromia, Ethiopia.

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ABBREVIATIONS

AHA	Acute Hemolytic Anemia
ATP	Adenosine Triphosphate
BC	Before Christ
DBS	Dried Blood Spot
EDTA	Ethylene Diamine Tetra acetic Acid
EHNRI	Ethiopian Health &Nutrition Research Institute
Fok I	Enzyme from Flavobacterium okeanokoites
FST	Fluorescent Spot Test
Gd	G6PD coding gene
Gd^-	G6PD coding mutated gene
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
G6PD (+)	Glucose-6-phosphate dehydrogenase normal
G6PD-D	Glucose-6-phosphate dehydrogenase deficiency
G6PD Med.	Glucose-6-phosphate Dehydrogenase Mediterranean
GSH	Reduced glutathione

GSSG	Oxidized glutathione
GSHPX	Glutathione Peroxidase
Hb	Hemoglobin
HMP	Hexose monophosphate pathway
HZ	Hertz
H_2O_2	Hydrogen peroxide
Kb	Kilo base
6-PG	6- Phosphogluconate
KDa	Kilo Daltan
Km	Michael's constant
MboII	Enzyme from *Moraxella bovis*
MIS	Malaria Indicator Survey
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	(Reduced) Nicotinamide Adenine Dinucleotide Phosphate
Nla III	Enzyme from *Neisseria lactamica*
NMG	National Malaria Guidelines

PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PPP	Pentose Phosphate Pathway
PQ	Primaquine
RBC	Red blood cell
RFLP	Restriction Fragment Length Polymorphism
Rpm	Round per minutes
SH	Sulfhydrl
TBE	Tris-Borate-EDTA
TNBT	Tetra Nitro Blue Tetrazolium
UV	Ultraviolet
V	Volts
WHO	World Health Organization

1. INTRODUCTION

1.1.Background of the Study

Glucose-6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme in red blood cells which catalyzes the first step in pentose phosphate pathway (PPP). Through a series of reactions, PPP converts glucose-6-phosphate to ribose-5- phosphate, a precursor of many important molecules such as RNA, DNA, ATP, CoA, Nicotinamide Adenine Dinucleotide (NAD), and Flavine Adenine Dinucleotide (FAD) (Noori-Daloii et al., 2008). PPP in the presence of G6PD also produces Nicotinamide Adenine Dinucleotide Phosphate (NADPH) molecules which act as an electron donor, which is a key hydrogen donor for the reduction of oxidized glutathione (GSSG) to a tri-peptide known as reduced glutathione (GSH). This tri-peptide is used as a reducing agent by glutathione peroxidase, which is involved in the detoxification of hydrogen peroxide (Ondei, et al., 2009).

The G6PD enzyme is essential for maintaining the integrity of the erythrocytes, preventing the oxidation of hemoglobin and other cellular proteins through providing reducing power in the form of NADPH to all cells including mature red blood cells, where the absence of mitochondria restricts the production of NADPH to PPP (Aboud, 2012). G6PD gene is linked to X-chromosome. Females can thus be homozygously deficient or heterozygously deficient, whereas males are hemizygously deficient. Heterozygously-deficient women have a mixed population of erythrocytes. One of the erythrocyte populations is G6PD deficient; the other has normal G6PD function (Peters, and Van Noorden 2009). Although G6PD is a house keeping enzyme that is expressed in all tissues, clinical manifestations of its deficiency are seen almost exclusively in red blood cells (RBC) including: neonatal jaundice and acute hemolytic anemia related to drugs, infection, or the ingestion of fava beans (Al-Musawi et al., 2012).

G6PD deficiency is the commonest clinically significant enzymopathy in humans. More than 400 million people worldwide are affected by G6PD deficiency which may cause: favism, drug-induced acute hemolytic anemia, severe chronic non-spherocytic hemolytic anemia (CNSHA), neonatal jaundice, and hemolytic anemia associated with viral or microbiological infections **(**Minucci et al., 2009). The highest prevalence of G6PD deficiency mainly regards to tropical Africa, the Middle East, tropical and subtropical Asia, Papua New Guinea, and various Mediterranean regions. In these geographic areas, G6PD deficiency may represent a selective advantage due to the increased resistance to severe *Plasmodium falciparum* infection of the affected individuals **(**Minucci et al., 2009).

G6PD deficiency is characterized by abnormally low levels of G6PD activity. Individuals with diminished G6PD activity are susceptible to cellular oxidative damage and can exhibit symptoms including haemolytic anemia and jaundice in response to a number of causes, most commonly infection or exposure to certain medications. In particular, treatment with antimalarial drugs such as those in the 8-aminoquinolone group (e g, primaquine, pamaquine and tafenoquine, etc) can cause acute haemolysis in people with G6PD deficiency. Currently Primaquine is the only drug available that is capable of radical cure of *Plasmodium vivax* (Kahn et al., 2013).

G6PD-deficient red blood cells (RBCs) have been deeply characterized, because the pentose phosphate pathway is the unique source of NADPH, which enables RBCs to counter balance the oxidative stress triggered by several oxidant agents preserving the reduced form of glutathione (GSH). In normal RBCs the ratio between reduced and oxidized GSH is 100:1. If NADPH concentrations cannot be maintained, as in Glucose-6-phosphate dehydrogenase deficiency (G6PD-D), the GSH levels fall and oxidative damage occurs resulting in an acute hemolysis (Minucci, et al., 2009).

In sub-Saharan Africa, 3 variants occur with polymorphic frequencies above 1%: wild type G6PD B, a non-deficient variant G6PD A and the deficient variant G6PD A- type (Carter et al., 2011; Carine 2012). The non deficient variant G6PD A type results from a point mutation 376A→G in exon 5 whereas the deficient variant G6PD A- has a 376A→G mutation and an additional one 202G→A (at the same time) in exon 4 in the G6PD gene (Abdoul Karim et al., 2014). Mediterranean type was spread to Middle East and North Africa (Al-Allawi et al., 2010). In this study we have characterized the G6PD deficiency by PCR-RFLP techniques in samples collected from Oromia, Gambella and Benishangul Gumuz malaria endemic areas. Individuals from different malaria endemic areas were selected to determine the frequency of mutation in the study populations.

1.2.Statement of the Problem

Although G6PD deficiency is part of general health screening in many countries, this enzymopathy has received little attention in Ethiopia during application of anti-malarial drugs to control malaria transmission. G6PD A-, the commonest African type mutation has been assumed to be the major variant associated with G6PD deficiency.

1.3. Objective

1.3.1 General Objective

The main objective of this study is to identify and determine the frequency of allelic variants of Glucose -6- phosphate dehydrogenase (G6PD) in malaria endemic areas of Oromia, Gambella and Benishangul Gumuz regions in Ethiopia.

1.3.2. Specific Objectives:-

1. To determine the prevalence of Mediterranean 563C→T mutation
2. To determine the prevalence of non deficient African A (A376G) and
3. To determine the prevalence of deficient African A- (A-376-G/G-202-A) mutation

1.3. Significance of the Study

This study would provide information about the distribution and frequency of Glucose-6-phosphate Dehydrogenase Deficiency (G6PD-D) in selected malaria endemic area in Ethiopia, which would be helpful for evidence-based use of drugs, like primaquine as part of national strategy that could greatly accelerate the minimization of malaria transmission in Ethiopia.

2. LITRATURE REVIEW

2.1. Background

Glucose-6-phosphate dehydrogenase (G6PD) is a ubiquitously expressed enzyme that has a housekeeping role in all cells, and is particularly critical to the integrity and functioning of red blood cells (RBCs). The G6PD gene has many mutant alleles which entail a decrease in enzyme activity, expressing the G6PD deficient phenotype. This trait is widespread in many human populations in whom several of the underlying mutant alleles are present at variable polymorphic frequencies (Howes et al., 2013a).

G6PD deficiency selectively affects RBCs for two reasons. The most known mutations cause a decreased stability of the enzyme, and since these cells do not have the ability to synthesizes proteins, the enzyme level decreases as cells age during their 120 days lifespan in circulation. Second, RBCs are delicately susceptible to oxidative stress from exogenous oxidizing agents in the blood as well as the oxygen radicals continuously generated as haemoglobin cycles between its deoxygenated and oxygenated forms. When G6PD activity is deficient, RBC has a diminished ability to withstand stress, and therefore causes haemolysis (Howes et al., 2013a).

In metabolic maps, G6PD is commonly referred to as the first enzyme of pentose phosphate pathway. It is now clear that the main role of G6PD is not glucose utilization rather; it is production of the reduced form of NADPH. NADPH, produced in the reaction catalyzed by the X-linked glucose - 6 -phosphate dehydrogenase in the PPP plays a key role in supplying reducing equivalents in red blood cells. G6PD protects cells against highly reactive oxygen derivatives (Samtani, 2013).

The highly reactive oxygen radicals either decay spontaneously or converted by superoxide dismutase to hydrogen peroxide (H_2O_2), which is still highly toxic to cells. Detoxification of H_2O_2 to H_2O and O_2 is effected by catalase and by glutathione peroxidase (GSHPX). NADPH is crucial for the function of both enzymes; it is a structural component of catalase and it is required as a substrate by glutathione reductase, which regenerates GSH when it has been oxidized to GSSG by GSHPX. G6PD deficient red blood cells are highly vulnerable to oxidative damage, even though G6PD deficiency is never complete in humans (Luzzatto et al., 2009).

Glucose 6-phosphate dehydrogenase deficiency (G6PD-D) is one of the most common inherited disorders in human so that more than 400 million people are affected worldwide (Cappellini, et al., 2008, Nezhad et al., 2009). G6PD deficiency is caused by defects in G6PD gene and results in a number of different hemolytic anemia due to exposing to some oxidative agents. This enzyme catalyses the first step of the pentose phosphate pathway and provides cells with required NADPH for biosynthesis and protecting them against oxidative stress. G6PD is the only NADPH generating enzyme of RBCs and the most important function of this enzyme is detoxification of oxidative agents, so RBCs are much more sensitive to the lack or deficiency of this enzyme rather than other tissues (Nezhad et al., 2009).

G6PD mutations occur in coding region and mainly result in single amino acid substitutions (Nezhad, K., et al., 2009). The G6PD gene exhibits remarkable polymorphism in human populations and is known to have over 400 variants. These variants are distinguished by their electrophoresis and biochemical characteristics and some variants are not associated with significantly reduced enzyme activity in erythrocytes (Hue Thi et al., 2009).

G6PD deficiency was first discovered by investigating a possible genetic basis for sensitivity to primaquine (Luzzatto et al., 2009). Glucose-6-phosphate Dehydrogenase (G6PD) deficiency was discovered by Alving and coworkers when they investigated the unusual haemolytic reaction that occurred in ethnic black individuals following the administration of primaquine, an 8-aminoquinoline, for the radical treatment of malaria in 1960's. Since that time, numerous other drugs were reported as being potentially harmful in G6PD-D individuals. There is no relationship in chemical structure among all of these drugs, but they have in common the ability to stimulate the pentose phosphate pathway in red blood cells, which mean that they are able to oxidize NADPH, directly or indirectly (Luzzatto et al., 2009).

2.2. The Genetics of G6PD

The G6PD gene (*Gd)* is located near the telomeric region of the long arm of the X chromosome (band Xq28); close to the genes for hemophilia A, congenital dyskeratosis, and color blindness (consisting of 13 exons and 12 introns) as shown in Fig. 1. G6PD gene encodes 515 amino acids and has GC-rich (more than 70%) promoter region (Cappellini, et al., 2008; Luzzatto et al., 2009; Minucci, 2009; and Phompradit, et al., 2011). X-linkage of *Gd* has three major consequences: (1) *Gd* mutations display the typical pattern of Mendelian X-linked inheritance; (2) severe G6PD deficiency is much more common in males than in females; and (3) as a result of X-chromosome inactivation, females heterozygous for two different *Gd* alleles exhibit somatic cell mosaicism. This means that if one of the alleles entails enzyme deficiency, about half the cells will be G6PD (+) and the other half will be G6PD (−) (Luzzatto et al., 2009).

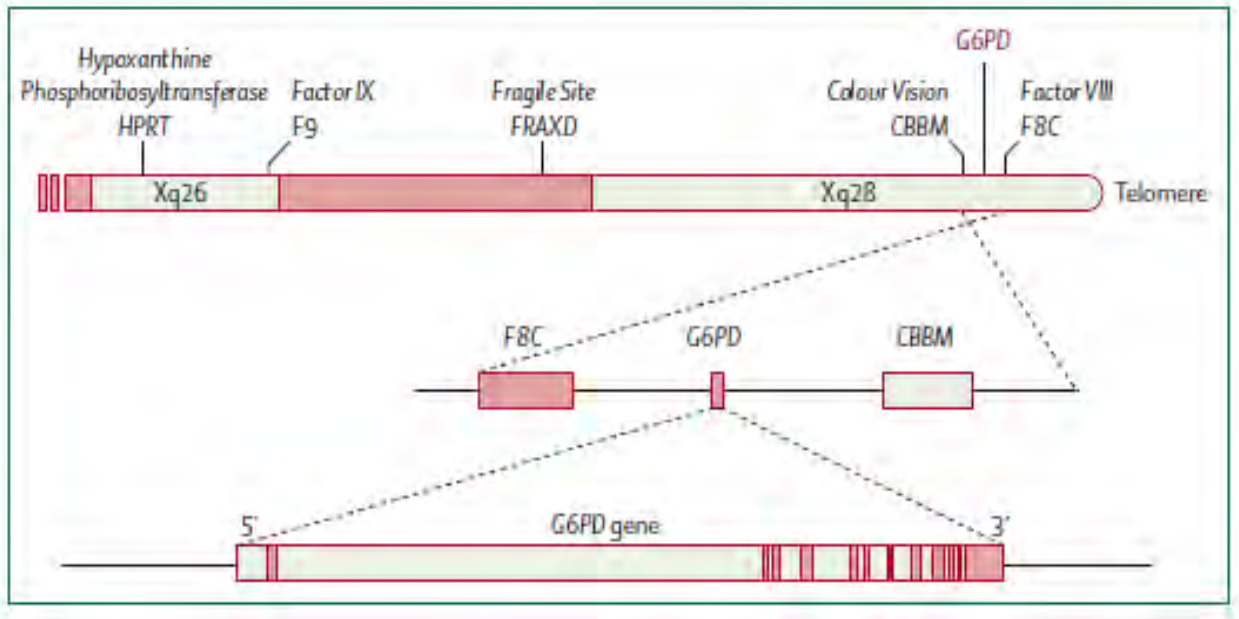

Figure1: Location of G6PD gene on X-chromosome (Cappellini, et al., 2008).

G6PD enzyme consists of either dimer or tetramer forms of a protein subunit consisting of 514 amino acids. Each subunit binds to $NADP^+$ molecule for its structural stability, which are positioned close to the interface where the two subunits of each dimer bind (Au SWN et al., 2000). The majority of mutation disrupts the enzyme structural stability and thus reduces its overall activity. The effect of each mutation on enzyme structure and function depends on the location of the substituted amino acid. For example, many of the most severe mutations map to exon 10 which encodes the binding interface of the subunits and therefore disrupt its quaternary structure and stability. These mutations cause the most severe clinical symptoms and as such do not reach polymorphic frequencies; instead they usually result from independent spontaneous mutations. Mutations which do not cause such severe reductions in enzyme activity are widely distributed across the gene's coding region and throughout the enzyme structure, and have been found to reduce the efficacy of protein folding. The residual enzyme activity of G6PD variants ranges from 1% to 100% (Howes et al., 2013a).

G6PD activity decreases with cell age: it is estimated that in normal blood, reticulocytes have about five time's higher activity levels than the oldest 10% of RBCs (Luzzatto, 2006). The oldest cells are therefore most vulnerable to oxidative stress. In individuals with intrinsically reduced G6PD enzyme activity due to genetic mutations, the ageing process is effectively speed up, with larger proportions of cells having lower enzyme levels and being at increased risk of oxidative damage (Howes et al., 2013a).

2.3. Function and Structure of G6PD

G6PD catalyses the first reaction in the pentose phosphate pathway, in which glucose is converted into the pentose sugars required for glycolysis and for various biosynthetic reactions. The pentose phosphate pathway also provides reducing power in the form of NADPH by the action of G6PD and 6-phosphogluconate dehydrogenase. NADPH serves as an electron donor for many enzymatic reactions essential in biosynthetic pathways, and its production is crucial to the protection of red blood cells from oxidative stress. G6PD is also necessary to regenerate the reduced form of glutathione that is produced with one molecule of NADPH (Tsai., et al., 1998, Cappellini, et al., 2008). In this case the reduced form of glutathione is essential for the reduction of H_2O_2 and oxygen radicals and maintenance of haemoglobin and other red-blood-cell proteins in the reduced state (Ruwende et al., 1998).

A model of the three-dimensional structure of G6PD enzyme is presented in Fig. 2 (Kiani et al., 2007). The enzyme is active as a tetramer or dimer, in a pH-dependent equilibrium. Every monomer consists of two domains: the N-terminal domain (amino acids 27–200), with a β–α–β dinucleotide binding site (amino acids 38–44); and a second, larger, β+α domain, consisting of an antiparallel nine-stranded sheet (Cappellini, et al., 2008).

Viewing the structure, at 3 A (0·3 nm) resolution, reveals NADP+ (a coenzyme) molecule in every subunit of the tetramer, distant from the active site but close to the dimer interface. Stability of the active quaternary structures is crucial for normal G6PD activity (Cappellini, et al., 2008).

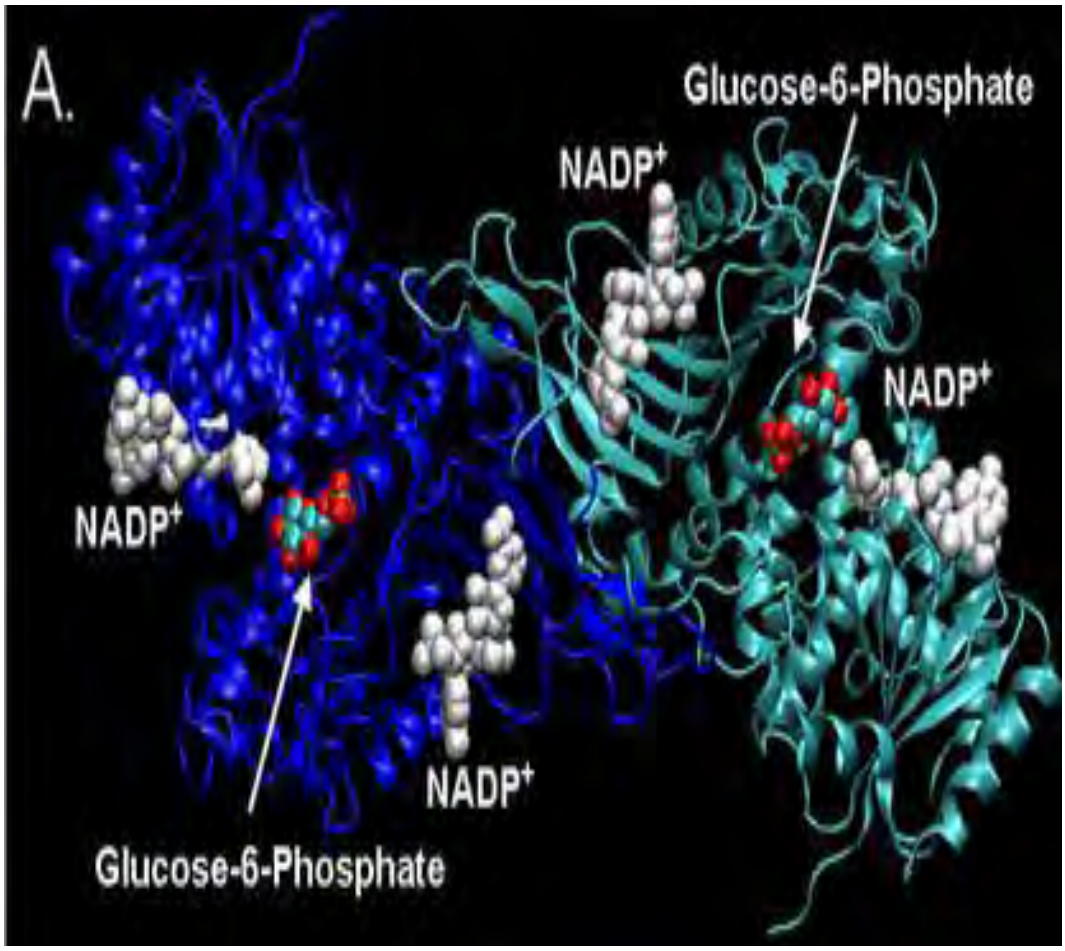

Figure 2: Three-dimensional model of active G6PD dimer (Kiani, et al., 2007)

2.4. Pentose Phosphate Pathway

Glucose-6-Phosphate Dehydrogenase (G6PD) is a key enzyme in the pentose mono-phosphate pathway and provides the NADPH essential for a number of biosynthetic and detoxifying reactions (Al-Allawi, et al., 2010). The first step of the pentose phosphate pathway is catalyzed by G6PD. In this step, NADP+ is reduced to NADPH, and ribulose-5-phosphate (Turner 2000). The second enzymatic step in this pathway is NADPH production as a consequence of reactions that reduce oxidized glutathione (GSSG) to reduced glutathione (GSH) as indicated in Fig. 3. The only defense against oxidant stress in the red blood cell (RBC) is GSH production. Therefore, pentose phosphate pathway's main function is the generation of reducing capacity through the production of NADPH and ultimately, GSH. This is essential for cell survival and is available in the erythrocyte for generating reducing capacity (Allahverdiyev et al., 2012).

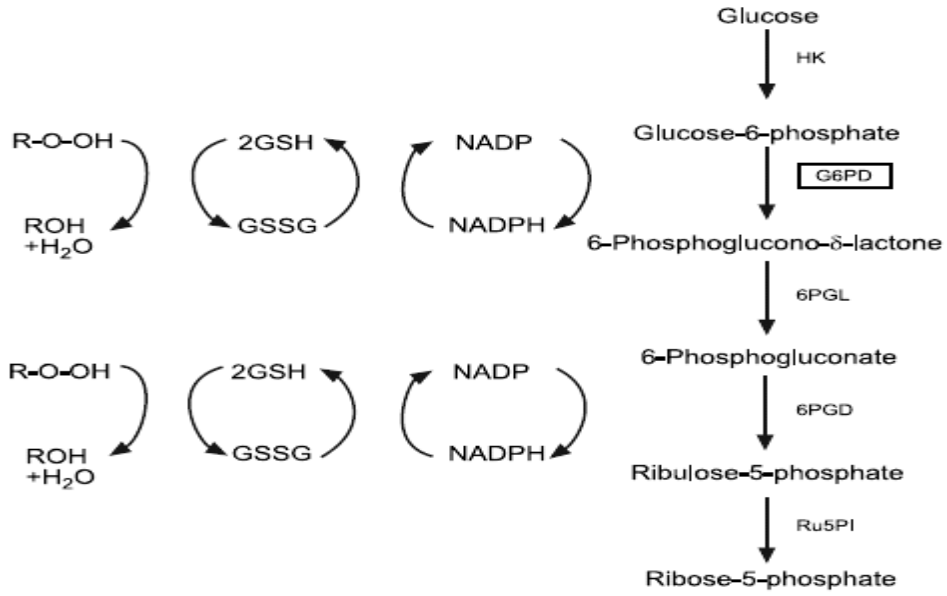

Figure 3:-The action of G6PD in the pentose phosphate pathway (PPP) (Mehta et al., 2000).

2.5. Nature of Mutations

G6PD-deficient subjects have different mutations in the coding region of the G6PD gene. All mutations of the *G6PD* gene that result in enzyme deficiency affect the coding sequence (Noori-Daloii et al., 2008) and these are indicated in Fig. 4 (Cappellini, et al., 2008). Most of the mutations are single-base substitutions leading to amino acid replacements. The current database of some 140 mutants consists, with few exceptions, of single mis-sense point mutations, resulting single amino acid replacements in the G6PD protein. The exceptions are small deletions (of one to eight amino acids), and a few instances in which two point mutations rather than one are simultaneously present (for instance, in G6PD A-, the variant most commonly encountered in Africa). In most cases, these mutations cause G6PD deficiency by decreasing the *in vivo* stability of the protein: thus, the physiological decrease in G6PD activity that leads to greatly accelerated red blood cell aging (Noori Daloii et al., 2008).

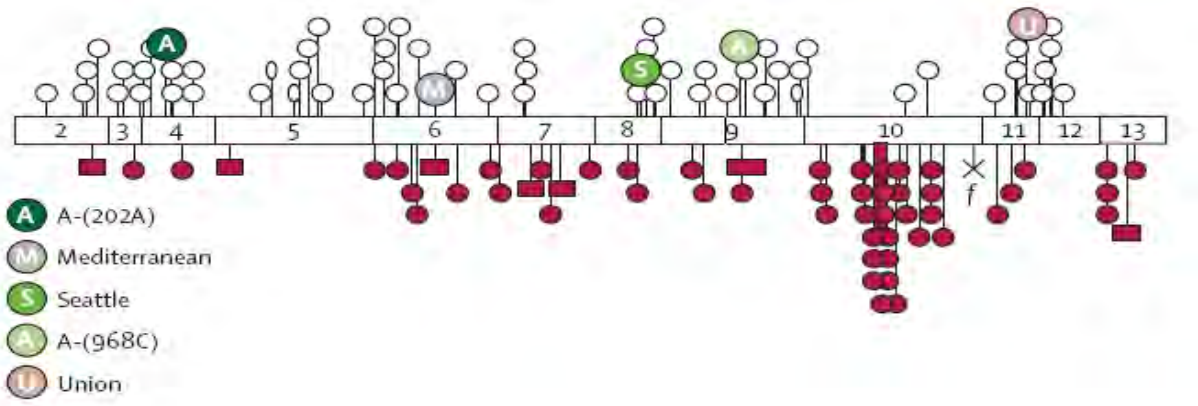

Figure 4: Most common mutations along coding sequence of *G6PD* gene (Cappellini, et al., 2008). (Regions indicated by numbers (2-13) shows exons at which G6PD mutation occurs and regions indicated by letters shows place where mutation happened and name of mutation i.e. A (A-(376/202)), M- Mediterranean type mutation, S- Seattle, A- mutation of (376/968) U- different types of mutation at exon 11 and 12 and red indicates mutation).

2.6. Molecular Epidemiology of G6PD Deficiency

It has been estimated that more than 400 million people worldwide are affected by G6PD-D (Lorenz et al., 2013). The highest prevalence is reported in Africa, southern Europe, the Middle East, Southeast Asia, and the central and southern Pacific Islands; however, because of migration, deficient alleles are nowadays quite prevalent in North and South America and in parts of northern Europe. In recent years, molecular analysis has been used to map the prevalence of G6PD-D (Cappellini, et al., 2008). G6PD variant gene frequencies range from 0.5% in Northern European populations, to over 25% in parts of central and West Africa, the Middle East and South East Asia. Due to migration patterns and ease of travel, G6PD-D may be encountered nowadays nearly in any corner of the globe **(**Aboud 2012).

Polymorphisms of the G6PD gene are numerous, with G6PD deficiency due to single point mutations, deletions, and insertions and, rarely, splicing variants; approximately 200 variant alleles have been described and 140 variants were characterized at DNA level. Phenotypically, G6PD-D is most often seen in hemizygous males. Heterozygous females display partial G6PD-D, consisting of normal G6PD and G6PD-D erythrocyte populations; homozygous females are less common. G6PD-D is thought to have been maintained in populations exposed to P. *Plasmodium* spp. for example, in two large case-control studies of more than 2,000 African children, the common African form of G6PD-D (G6PD A−) was associated with a 46−58% reduction in the risk of severe malaria for both female heterozygotes and male hemizygotes. The mechanism for this protective effect may be the higher sensitivity of G6PD-D erythrocytes to hydrogen peroxide produced by the parasite. This causes erythrocyte damage that impairs parasite growth and/or leads to early erythrocyte phagocytosis (Beutler et al., 2007).

2.7. Features of G6PD in Red Blood Cells

Biochemical evidence showed that the G6PD protein in red blood cells is the same as that in other somatic cells; thus, when red blood cells are severely deficient in G6PD, this deficiency is also found in other somatic cells. However, a significant difference in the metabolism of G6PD arises from the characteristic inability of mature red blood cells to synthesize protein. In red blood cells any G6PD molecule undergoing denaturation/breakdown cannot be replaced. In normal red blood cells, the decay of G6PD approximates an exponential with a half-life of about 60 days. The age dependence of red blood cells G6PD activity is so characteristic that it can almost be regarded as a marker of red blood cells age. In normal blood, reticulocytes have about five times more activity than 10% the oldest red blood cells (Luzzatto et al., 2009).

2.8. Population Variants of G6PD and *Plasmodium falciparum* Malaria

G6PD deficiency became an apparent trait that is found mainly in populations originating from tropical and subtropical areas of the globe. The geographic distribution was similar to that of P. *falciparum* malaria and this suggested that G6PD deficiency owes its distribution to selection by malaria parasite. *Plasmodium* requires non-protein glutathione (GSH) for growth. Since GSH is reduced in G6PD deficiency, proliferation of *Plasmodium falciparum* parasite might be reduced in the enzyme deficiency (Samtani, 2013).

The correlation between the worldwide distribution of G6PD deficiency and that of *Plasmodium falciparum* prompted formulation of the "malaria hypothesis" nearly half a century ago. Since that time, numerous studies (micro mapping), as well as clinical studies, have supported the concept that G6PD deficiency confers some degree of resistance to the potentially lethal malaria parasite, *P. falciparum,* and that in malaria-endemic areas, G6PD alleles associated with enzyme

deficiency have therefore been subjected to positive Darwinian selection. Three large controlled clinical studies, all carried out in Africa, showed concordantly that children with G6PD deficiency tend to have less severe malaria and, therefore, presumably a decreased risk of dying of malaria. However, all the three studies (from Nigeria, Gambia and Kenya and Mali) agree in supporting the concept of malaria selection for G6PD deficiency (Luzzatto et al., 2009).

Individuals with G6PD-D have an evolutionary survival advantage in malaria-endemic regions as G6PD deficiency confers a degree of protection against severe malaria. The selective advantage of G6PD-D under malaria pressure has left its mark in the human genome as a "selective sweep" surrounding the G6PD gene. Not surprisingly G6PD-D is most prevalent in regions where malaria was prevalent (Lorenz et al., 2013).

When a gene that has some potential for decreasing fitness achieves a high frequency in some populations, it is necessary to assume that in those populations it also confers a survival advantage. Thus, a balance has been achieved between the advantage and the disadvantage conferred by a gene, and this is designated a balanced polymorphism. The mortality caused by malaria in some parts of the world is so high that a large number of genetic traits that defend against this infection have evolved in mankind, and many polymorphisms affecting the RBC seem to have reached high frequencies for this reason (Noori-Daloii et al., 2008).

2.9. Glucose-6-Phosphate Dehydrogenase Deficiency Classification

G6PD deficiency is the most common human metabolic disorder affecting more than 400 million people worldwide (Minucci et al., 2009). Individuals with this genetic disorder may exhibit non-immune haemolytic anaemia in response to a number of causes, most common infection or exposure to certain medications or *fava beans* **(**Peters and Van Noorden 2012). The severity of G6PD deficiency can be assessed according to a classification system of the WHO. Severe forms characterized by chronic nonspherocytic hemolytic anemia belong to class I. Class II includes G6PD variants with less than 10% of the normal G6PD function, described as intermediate form. The mild and most common variants belong to class III, presenting 10–60% activity compared with wild-type G6PD. Variants with 60–90% of normal G6PD function are referred to as class IV and represent the asymptomatic forms; and class V is greater than 110% (Stanton 2012).

2.10. Mechanism of Hemolysis

In erythrocytes, G6PD deficiency cannot form NADPH and unformed NADPH creates a deficiency in conversion of the oxidized form of glutathione (GSSG), to its reduced form (GSH). There is normally plenty of GSH in erythrocytes and it protects the cell from oxidizing agents. If G6PD is deficient, hemoglobin is oxidized by oxidative substances to be eliminated and it returns methemoglobin that cannot function normally. Also, hemoglobin precipitates with denaturation in the cytoplasm forms Heinz bodies. These structures attached to the membrane with disulfide bonds and disrupt its normal structure. Erythrocytes that contain Heinz bodies in their cytoplasm are sequestered by macrophages in the spleen and removed from the circulation (Howes et al., 2013a).

2.11. Clinical Manifestation of Hemolysis

2.11.1. Drug-Induced Hemolysis

Hemolytic anemia is the example of the clinical manifestation of G6PD deficiency. This is probably true, because of the history of how G6PD deficiency was discovered, i.e., through investigation of primaquine-induced hemolysis, and it may be in part because this type of hemolytic reaction is most easily defined and most easily studied (Beutler et al., 2007). A large number of drugs and other chemicals that may have the capacity to precipitate hemolytic reactions in G6PD-deficient individuals are listed in Table 1. Some drugs, such as chloramphenicol, may induce mild hemolysis in people with severe, Mediterranean-type G6PD deficiency (Samtani, 2013). Primaquine is one of many drugs that shorten RBC life span in G6PD deficient persons (Noori-Daloii, et al., 2008). Drugs that cause hemolysis in G6PD deficient persons impose oxidative damage to erythrocytes leading to erythrocyte destruction. Hemolysis typically occurs 24 to 72 hours after ingestion, with resolution within four to seven days (Iwai, et al., 2003).

Table 1: Drugs and chemicals that should be avoided by persons with G6PD deficiency

(Samtani, 2013)

Drugs/chemicals	**Remark**	**Drugs/chemicals**	**Remark**
Acetanilide	Chemical	Sulfacetamide	Anti-bacterial
Doxorubicin,	Antibiotics	Sulfamethoxazole	Drug
Furazolidone (Furoxone)	drug	Sulfanilamide	Antibiotics
Methylene Blue,	Chemicals	Primaquine	Treatment drug for malaria and Pneumocystis pneumonia.
Nalidixic acid (NeGram)	Antibacterial	Phenylhydrazine	Chemical
Naphthalene	Chemical	Sulfapyridine	Antibiotics
Niridazole (Ambilhar)	Drug	Thiazolesulfone	Antibacterial
Nitrofurantoin/Furadantin	Antibiotics	Toluidine blue	Chemical (dye)
Phenazopyridine/Pyridium	Anti pains	Trinitrotoluene	Chemical (explosive)

2.11.2. Infection-Induced Hemolysis

Infection is probably the most common cause of hemolysis in subjects with G6PD deficiency. Numerous bacterial, viral and rickettsial infections have been reported as precipitants, but particularly important infections are infectious hepatitis, pneumonia and typhoid fever (Cappellini et al., 2008, Howes et al., 2013). Viral infections of the upper respiratory and gastrointestinal tracts are reported to cause more severe hemolysis than bacterial infections in G6PD-deficient children (Cappellini et al., 2008). The mechanism of infection-induced hemolysis is not clear, but it is thought to be that during the infection, superoxide anion and H_2O_2 production by macrophages cause the hemolysis (Howes et al., 2013a).

2.11.3. Favism

Favism is an illness that occurs in G6PD deficiency individuals with acute hemolysis by eating raw faba beans, Wet, dry or frozen fava bean ingestion of faba beans, even by the mother eats fava beans may cause hemolysis to the new born infants while breast feeding (Allahverdiyev, et al., 2012). The fact that exposure to fava beans (*Vicia fava*, broad bean) is toxic and potentially fatal for some individuals has been known since the time of the old Greeks (Noori-Daloii et al., 2008). In favism, damage in erythrocytes is similar to oxidative damage of drugs. Fava beans contain vicine, convicine and ascorbic acid which have oxidative properties. The most commonly cited convisine and visine glycosides generated during digestion of fava beans by β-glycosidase or acid hydrolysis, which are converted to "divicine" and "izouramil." Divicine and izouramil reduce the level of the NADPH and GSH as a result damage the cell membrane by the formation of cross-connection with Heinz bodies (Allahverdiyev, et al., 2012).

2.12. Some Important G6PD Variants

More than 400 variants of G6PD have been distinguished based on their biochemical characteristics, enzyme kinetics, physicochemical characteristics, and other parameters. G6PD wild type is the most commonly found enzyme type and it is used as a standard for normal enzyme activity and electrophoretic mobility.

2.12.1. African Mutations

In sub-Saharan Africa, 3 variants occur with polymorphic frequencies above 1%: wild type G6PD B, a non-deficient variant G6PD A and the deficient variant G6PD A-. The variant G6PD A results from a point mutation A376G in exon 5 whereas the deficient variant G6PD A- has an A376G mutation and an additional one G202A in exon 4. Other deficient variants associate the mutation A376G are: A542T (exon 6), G680T (exon 7) and T968C (exon 9) in the G6PD gene (Abdoul Karim et al., 2014).

G6PD A is the most widely seen variant worldwide and also the first variant in which the nucleotide mutation and amino acid substitution were determined. This Class IV variant has 90% of the enzyme activity of G6PD B^+. This variant also called the African variant because widely seen in Africa; 20-40% of African men and 20% of Africa-American men have this variant. It is faster than G6PD B+ electrophoretically and it does not cause hemolysis. G6PD A is derived from a single amino acid substitution of aspartic acid (GAU/GAC codons) to asparagine (AAU/AAC codons) at amino acid number 126, and this was the result of an adenine to guanine mutation at nucleotide number 376 (Allahverdiyev et al., 2012).

The other African variant is G6PD A- which is a Class III variant that has 10 to 60% of the activity of G6PD B+. The G6PD A- is a peculiar genotype determined by the concomitant presence of A376G plus G202A mutations (Minucci, et al., 2009), and 11% of African American men have this variant. The common mutation being at nucleotide number 202 is a result of a guanine to adenine mutation at amino acid number 68 substitution of valine (GUG codon) to methionine (AUG codon) (Allahverdiyev, et al., 2012).

2.12.2. Mediterranean Mutations

The G6PD Mediterranean is among the most important and prevalent variant of the enzyme, that is a result of C to T transition at nucleotide 563 (exon 6), causing acute hemolytic anemia (Nezhad et al., 2009). Mediterranean mutation C563T is among most common variants that lead to enzyme deficiency and often associated with Favism. The best known G6PD-deficient variants that occur at a high frequency are the African variant G6PD A- and the Mediterranean variants **(**Aboud 2012). Mediterranean variant is genetically polymorphic; it means that several different mutations cause this variant. One of its different types is caused by the mutation that converts nucleotide 563C of G6PD gene to T, as a result of which at position 188 of the polypeptide chain of enzyme, phenylalanine (TTC codon) substituted with serine (TCT codon) amino acid. With this replacement, the enzyme activity will be reduced to less than 10 percent of normal (Nakhaee et al., 2012).

2.13. Test for G6PD Deficiency

Various tests can be used for the detection of G6PD deficiency, which are based on the assessment of the NADPH production capacity of G6PD. The most frequently used tests that measure NADPH production are the fluorescent spot test, cytochemical assay and spectrophotometric assay. However, fluorescent spot test and the spectrophotometric assay are not reliable for the detection of heterozygous females. In addition, DNA analysis can be done to detect G6PD deficiency for the homozygous, hemizygous, and heterozygous-deficient patients. However, it requires to design primers for all mutations (Peters, and Van Noorden, 2009).

2.13.1. Test of G6PD Deficiency by DNA-Based Methods

Molecular methods use variant-specific primers to identify the presence or absence of specific mutations. These direct methods overcome uncertainties associated with variable enzyme activity cut-offs, anaemia, reagent breakdown and subjective classifications and also female heterozygotes will not be dangerously misclassified as G6PD normal (Howes et al., 2013a*)*. DNA tests can be used for the diagnosis of G6PD deficiency. In these extremely reliable tests, primers are used to check whether the G6PD gene contains a mutation (Lin et al., 2005, Ko et al., 2006). This can be used for the diagnosis of homozygous, hemizygous, and heterozygous deficient patients (Peters, and Van Noorden 2009).

2.13.2. Cytochemical Staining Assay

The Cytochemical staining assay is based on the intracellular reduction of the tetra nitro blue tetrazolium (TNBT) by the G6PD via exogenous electron carrier 1-methoxyphenazine methosulfate and TNBT is reduced to dark-colored water-insoluble formazan, which can be determined by light microscopy (Allahverdiyev, et al., 2012). Dark-purple granules are present in RBC that contains G6PD activity, whereas G6PD-deficient RBC remains unstained. The number of both positive (RBC with dark-purple) and negative stained cells (RBC with little or no staining) were counted at least 1,000 RBCs (Nantakomol et al., 2013). Cytochemical assay is reliable for detection of G6PD deficiency in heterozygous women but this is a relatively complicated test that requires a number of steps (Peters and Van Noorden 2009).

2.13.3. Fluorescent Spot Test (FS-test)

This technique is based on the visual evaluation of fluorescence reduced NADPH when activated by UV light. The method detects the fluorescence of NADPH, which is proportional to G6PD activity, under UV light long wave (365 nm). The FS test was performed on the heparinized blood using a commercial kit (R&D Diagnosis, Holargos, Greece). Ten microlitres of blood was first incubated with 200µl of the reagent mixture and spotted on to filter paper. Fluorescence intensity was measured at the beginning (zero time), 5, 10, and 20 min after incubation of blood with reagent mixture. Fluorescence intensity was classified into two groups: normal activity (bright fluorescence) and deficiency (no fluorescence) (Nantakomol et al., 2013).

2.13.4. Spectrophotometric Assay

The G6PD activity is measured based on the evaluation of absorbance at 340 nm given by NADPH formation; the quantitative evaluation is made by adding a precise amount of hemolysate to an assay mixture containing the substrate (glucose-6-phosphate) and its cofactor NADP; the rate of NADPH generation is spectrophotometrically measured at wavelength of 340 nm. (Minucci et al., 2009).

2.14. Management of G6PD Deficiency

2.14.1. Prevention and Treatment

The acute hemolytic anemia by G6PD deficiency in previously screened subjects is largely preventable by avoiding exposure to triggering factors. Of course, the practicality and cost-effectiveness of screening depends on the prevalence of G6PD deficiency in each individual community. Favism is entirely preventable by avoiding eating fava beans. Prevention of drug-induced hemolysis is also possible in most cases by choosing alternative drugs (Luzzatto, 2006). Once the diagnosis of G6PD deficiency has been made, prevention is the mainstay of management. The parents should be counseled regarding the pattern of inheritance, avoidance of oxidants risk of hemolysis during infection. Other family members at risk, including parents and siblings, should be screened **(**Aboud 2012).

The main treatment for G6PD deficiency is avoidance of oxidative stressors. Rarely, anemia may be severe enough to warrant a blood transfusion. Splenectomy generally is not recommended. Folic acid and iron potentially are useful in hemolysis, although G6PD deficiency usually is asymptomatic and the associated hemolysis usually is short-lived. Antioxidants such as vitamin E and selenium have no proven benefit for the treatment of G6PD deficiency. Research is being done to identify medications that may inhibit oxidative- induced hemolysis of G6PD-deficient red blood cells (Jennifer, et al., 2005).

3. MATERIALS AND METHODS

3.1. Study Area and Period

The study samples were collected during Malaria Indicator Survey (MIS) 2011, a nationally representative household survey conducted in malaria endemic areas of Ethiopia. Malarious areas are classified as areas below 2,000m mean sea level, and malaria epidemic prone areas, defined as areas between 2,000m and 2,500m mean sea level. Of the 12,000 dried blood samples collected during the survey, 2000 samples were selected randomly proportional to regional population size. This study analyzes a total of 620 samples (326 Female and 294 Male) that belongs to Oromia, Gambella and Benishangul Gumuz regions indicated in table 2.

Table 2 Sample Size taken from Oromia, Gambella and Benishangul Gumuz Malaria Endemic Areas by region and sex.

Regions	Total number of samples	**sex**	
		Number of Male	Number of female
Oromia	530	248	282
Benishangul Gumuz	52	24	28
Gambella	38	22	16

3.2. Laboratory Methods

A standard polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used for DNA genotyping: DNA was extracted from DBS using Qiagn DNA blood mini kit, conventional PCR was used for the amplification of DNA and three sets of endonuclease enzymes namely *fok I, NlaIII* and *Mob II* was used for restriction.

3.2.1. DNA Extraction

Genomic DNA was extracted from blood samples using a QIAamp DNA blood mini kit (Abdoul Karim et al., 2014). A single 3-mm DBS from each sample was transferred to a fresh 1.5-mL microfuge tube. Using the QiaAmp Mini Spin Kit (Qiagen, Hilden, Germany). In brief, the DBS was incubated for 10 minutes at 85°C in buffer ATL and briefly centrifuge to remove drops from inside the lid, then 20µl proteinase K solution was added. The sample was vortexed and incubated for 1 hour at 56°C to facilitate enzymatic digestion of the sample. Buffer AL was then added, the sample was incubated at 70°C for 10 minutes, absolute ethanol was added, and the sample was vortexed thoroughly to mix. The supernatant solution was applied to the QIAamp mini spin column and was bound to the columnviaa1-minute centrifugation at 8000 rpm for 1 minute. Filtrate was discarded, and the sample was washed on the column by the application of buffer AW1, followed by a 1-minute 8000 rpm for 1 minute and spin where the filtrate was discarded. Buffer AW2 was then applied, the sample wasspunat13, 000rpm for 3minutes, the filtrate was discarded, and the spin column was centrifuged again for 1minute at 13,000 rpm to remove all trace ethanol. The DNA-containing column was then moved to a clean 1.5mL micro centrifuge tube, and 50mL of DNAase/RNAase free water (Qiagen) added to the column. The columns were incubated with water for 1 minute at room temperature and then centrifuge at 8000

rpm for 1 minute and concentrate the gDNA in a small end volume (www. QIAamp DNA and Blood Mini kit).

3.2.2. Polymerase Chain Reaction (PCR) for DNA Amplification

PCR method was adopted to amplify *Gd* gene from the DNA samples. Mediterranean and African mutations were chosen for molecular identification of *Gd* gene mutation in malaria endemic regions in Oromia, Gambella and Benishangul in Ethiopia. Gene specific forward and reverse primers were used for amplification specific regions of G6PD gene. DNA of total samples was amplified for detection of (563C→T) mutation, which is characteristic of G6PD Mediterranean variant (Alfadhli et al., 2005).

Thermo cycling was performed using F Med (5- AGCTCT GAT CCT CAC TCC CC-3) and RMed (3-AGAGTCCTCGGAGTGGACCGG-5) primers to amplify exon 6 and flanking regions, involving Mediterranean mutation. The PCR reaction was carried out for 34 repeats. Each repeat consisted the following temperatures with time: initial denaturation: 94 °C for 5 minutes, and 32 cycles started as follows: denaturation 94°C for 1 minutes, annealing: 57°C for 1 minutes and extension 72°C: for 1minute and final extension at 72°C for 5 minutes (Alfadhli et al., 2005). The PCR mixture for African type and Mediterranean type mutation contained 12.5µl of Promega master mix (*Taq* in), 10µm of 0.25µl of forward and reverse primers, 4µl nuclease free water, 8µl template DNA in a total of 25µl.

`Genomic DNA was first amplified using primers for A376G forward and reverse and all samples positive for A376G was then subjected to PCR amplification using primers for G202A, forward and reverse in table 3 (Samilchuk et al., 1999).

Table 3 primers for G6PD African A and A- type mutation

Type of mutation	Primer sequence	exons
African A^+	Forward, *5'-CCCAGGCCACCCCAGAGGAGA-3'*	5
376A-G	Reverse, *3'-GATACTCGCACAGGCCCCGGC-5'*	
African A^-	*Forward, 5'-CACCACTGCCCCTGTGACCT-3'*	4
202G-A	*Reverse, 3'-TTCCACCCACCACAGTCCCGG-5'*	

Thermocycling was performed by *BIORAD icycler thermocycle system* for 34 repeats (each repeat consisted the following temperatures with time initial denaturation, denaturation, annealing, extension and final extension indicated in the table 4 (Samilchuk et al., 1999).

Table 4 Thermo cycle condition for G6PD African type mutation A+ and A-

Step	Temperature (°C)	Duration	Description	# of cycle
1	94	5 minutes	Initial denaturation	1x
2	94	45 seconds	Denaturation	32x
3	64	30 seconds	Annealing	
4	72	45 seconds	Extension	
5	72	5 minutes	Final extension	1x
6	4		Holding	1x

3.2.3. Sample Preparation for RFLP Analysis

3.2.3.1. Digestion

PCR product of G6PD gene for identification of 563C-T, A376G and G202A mutation was digested with *MboII, FokI, and NIaIII* restriction enzymes (New England BioLabs) respectively with reaction mixture contained Nuclease free water 7.5µl, (Cutsmart buffer 10x) 2 µl, Restriction enzyme 0.5µl, and PCR template 8µl, in Total of 20µl for 1h at 37°C, and inactivate the enzyme for 25 minutes at 65°C and the digested products was analyzed on 2% agarose gel. The G6PD-Med mutation at base position 563 creates a *MboII* site in exon 6, the African A376G mutation at base position 376 creates a *FokI* sites in exon 5 and the African G202A mutation at base position 202 creates *NIaIII* sites in exon 4 of the G6PD gene (Carine, et al., 2012).

Experimental controls are necessary to identify, understand and explain problems or inconsistencies in results (Al-Temmemy, et al., 2009). The following controls are commonly used in parallel with restriction enzyme (RE) digests: digested positive sample for African A376G and G202A, uncut (undigested) DNA, Positive control of PCR product (normal DNA), and Negative control (RE without DNA).

3.2.3.2. Gel Electrophoresis

A 2% agarose gel was prepared by melting 2g of agarose in 100ml of 1x TBE (Tris-Borate-EDTA) buffer in microwave oven for 3min. 10µl of Gel Red nucleic acid dye stain (GelRed is a sensitive, stable and relatively safe fluorescent nucleic acid dye (http://www.interchim.com); was added to melted gel and swirled to mix uniformly. The gel was casted to set in chamber with combs to make the wells (Dan Osei, 2013). 8µl of restriction enzyme digested DNA sample were mixed with loading dye (2µl) and was applied to the wells of the gel. The gel was run at 100 volts in electrophoretic gel system and photographed under ultraviolet (UV) visualization with gel documentation system.

4. RESULTS

Six hundred twenty (620) individual's (326 Female and 294 Male) blood sample was evaluated using PCR-RLFP techniques. Presence of DNA and PCR product of exon 5 was 308 base pairs, which was confirmed by gel electrophoresis in 2% agarose gel and comparing with DNA size marker shown in Fig. 5 and 6, respectively.

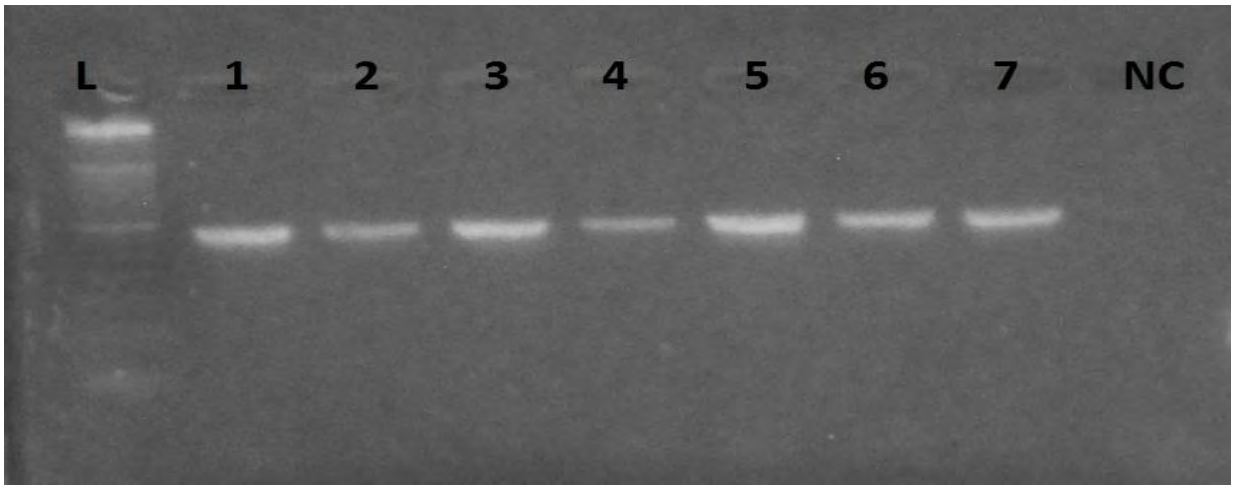

Figure 5. Electrophoretic pattern for DNA presence (L=Ladder, Lane 1-7 DNA sample, NC=Negative Control (all mixture without DNA sample).

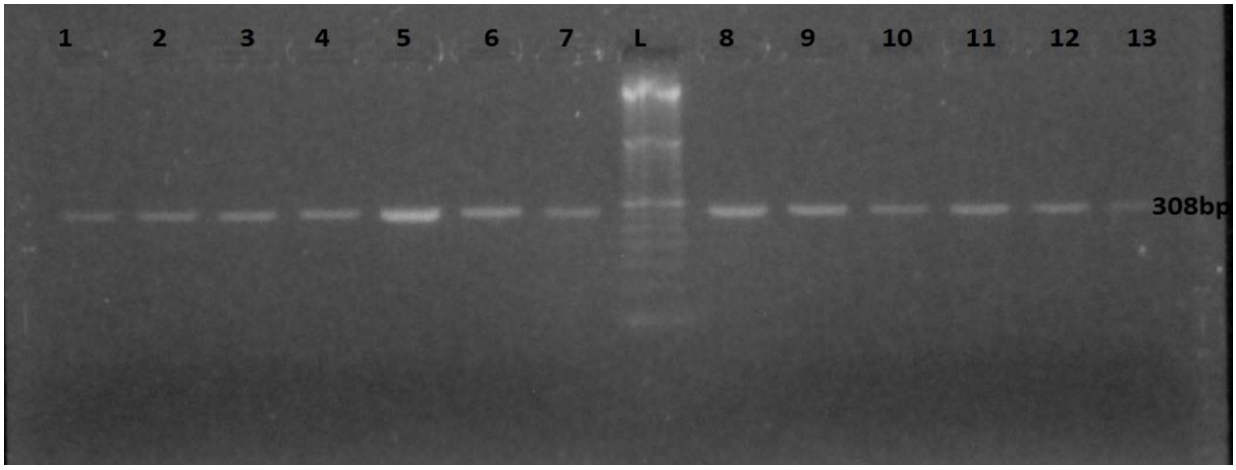

Figure 6. Electrophoretic pattern of PCR products of exon 5 (Lane 1-7 and 8-13 are DNA sample, L=Ladder).

The pattern of amplification and cutting of the 308 base pairs fragment for normal and mutant genes was indicated in Fig. 7 and 8 and table 5. African A type mutation creates an additional cut position in the amplified sequence, and 125bp and 183bp fragments was produced using *fokI* enzyme. African A type mutation was detected in 62 out of 620 samples with class IV deficient G6PD in Oromia, Benishangul Gumuz and Gambella regional states. Thus, the prevalence of this mutation was estimated to be 10% in Oromia, 10.5% in Gambella and 9.6% in Benishangul Gumuz regional states indicated in table 6.

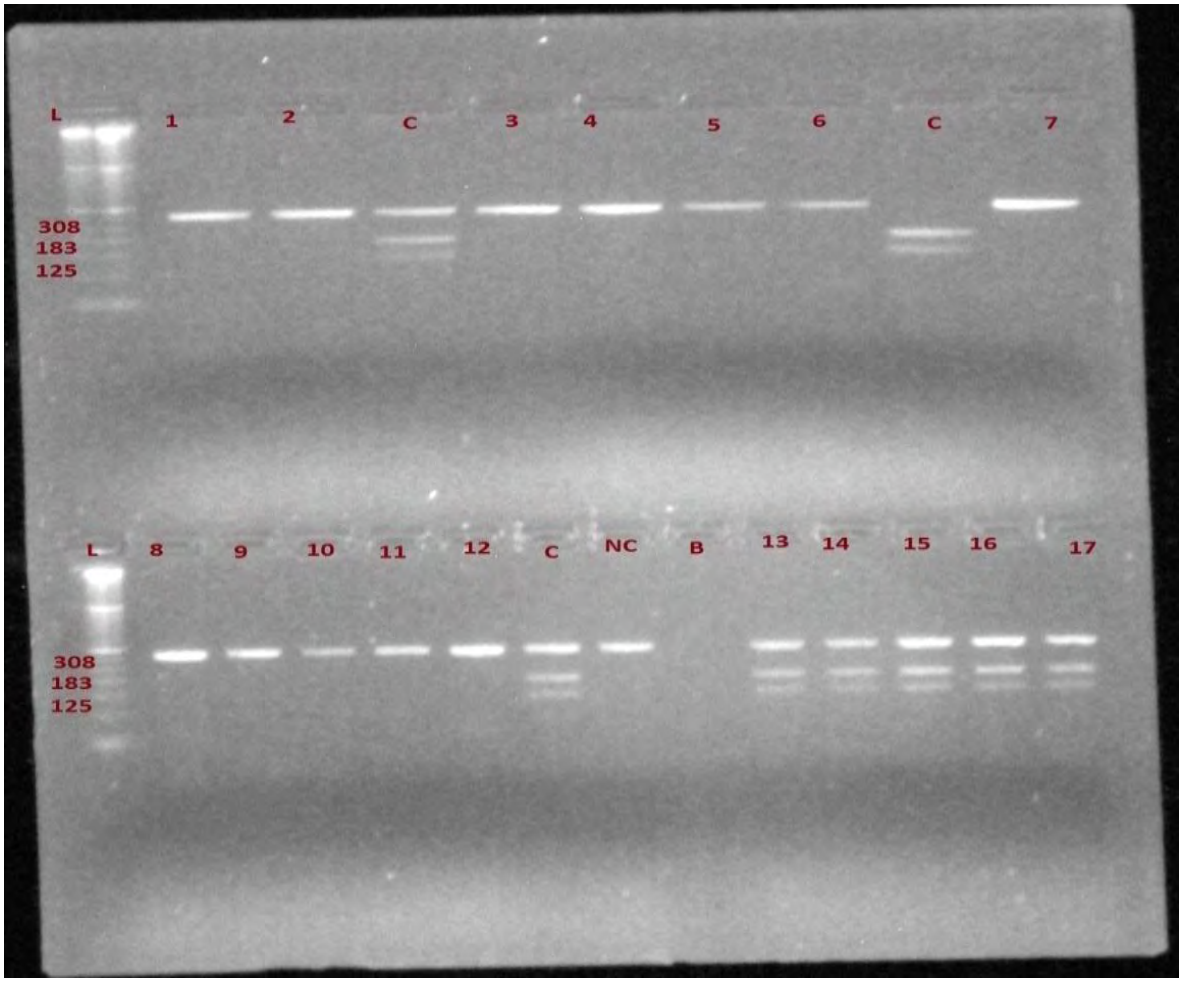

Figure 7. Electrophoretic pattern of African type A with *fokI* restriction enzyme (L=Ladder, DNA Sample 1, 2 , 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 are Negative sample, C= Positive control, NC= Negative control and B= is Blank.

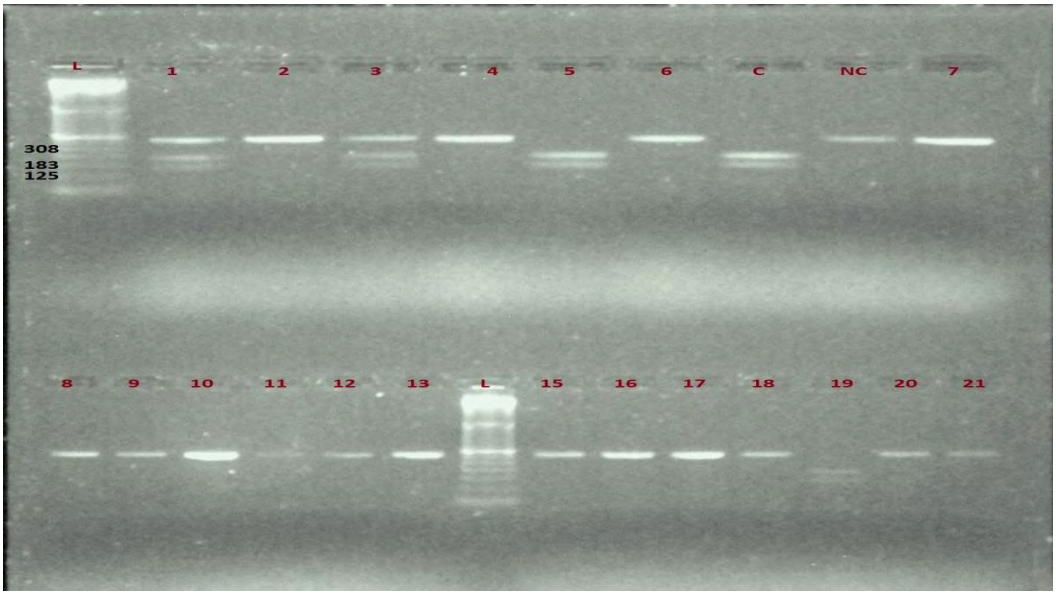

Figure 8 Electrophoretic pattern of African type A with *fok*I restriction enzyme (L= ladder, Sample 1-6, 7-13 and 15-21 are Negative DNA sample for African type A mutation, C= positive Control, NC=Negative control sample for African type A mutation). One (1) fragment represents negative for G6PD A type mutation (wild type), Two (2) fragments represents positive sample for G6PD A type mutation can be hemizygote male or heterozygote female).

Table 5. Restriction Analysis for G6PD A type mutation.

G6PD mutation	Amplified exon	Enzyme		PCR product, bp	Fragment size, Base pairs	
		Restriction enzyme	Restriction site		Wilde type	Digest mutant bp
African A	5	*FokI*	GGATG/ CCTAC	308	308	125, 183

Table 6. Distribution of G6PD mutation in Oromia, Gambella and Benishangul Gumuz regions.

Region	Total sample	Wilde type (B)	Mutant type (A)	% of mutant type (A)
Oromia	530	477	53	10
Benishangul Gumuz	52	47	5	9.6
Gambella	38	34	4	10.5
Total	620	558	62	10

4.1. Frequency of G6PD Deficient Genotypes

Genotyping of deficient variant after PCR-RFLP techniques indicated that out of 62 G6PD defective samples 40 females were heterozygous by three fragments, nine females are homozygous by two fragments and thirteen males are hemizygous also by two fragments indicated (Figs.7 and 8) for G6PD African A type variant in the samples from Oromia, Benishangul Gumuz and Gambella regions shown in Table 7 and 0.1 gene frequency. On the other hand Africa A- and Mediterranean types of gene mutation are not observed in this study.

Table 7 Frequency of G6PD genotypes among deficient subjects for African Atype

G6PD mutation	Types of genotypes	Numbers	distribution %
G6PD A	Heterozygous	40	64.51
	Homozygous	9	14.52
	Hemizygous	13	20.97
	Total	62	100

4.2. **Allele Frequency of African A type mutation**

G6PD A (376 A-G) was found to have a widespread distribution, accounting for allele frequency of 0.065 in Oromia, 0.063 in Benishangul Gumuz and 0.074 in Gambella as shown in Table 8.

Table 8 Allelic frequency of G6PD mutation among Oromia, Benishangul Ggumuz and Gambella regions of malaria endemic areas

Regions	No. of males		No.of females		Genotypes	Frequency of mutant alleles		Total numbers alleles)	Average frequency
	Normal	mutant	Normal	Mutant		Males	Females		
Oromia	237	11	240	42	11*,4†, 48‡	0.044	0.074	812	0.0652
B/G	24	0	23	5	4†,1‡	0.00	0.089	80	0.063
Gambella	20	2	14	2	2* ,1† 1‡	0.091	0.063	54	0.074

* Hemizygous male † Homozygous female and ‡ Heterozygous B/G Benishangul Gumuz

Positive samples for 376 A-G mutation in exon 4 and unknown G6PD Mediterranean type mutation (exon 6) was amplified and this PCR product was 211bp exon 4 and 285 base pairs from exon 6 which was confirmed by electrophoresis in 2% agarose gel and compared with DNA size marker presented in Fig. 9 and 10.

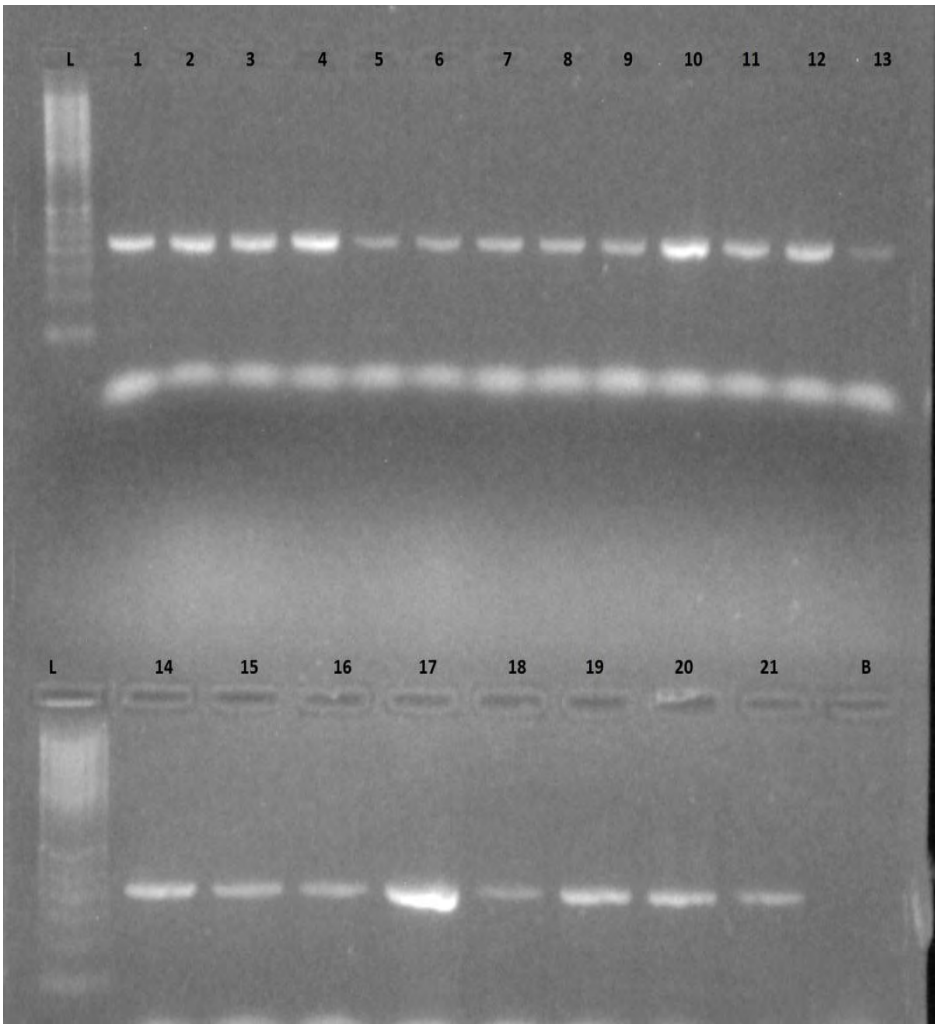

Figure 9. Electrophoretic pattern of PCR products for African type A- mutation (L=ladder, Lane 2-14 and 16-21 are amplified DNA sample and B= Blank (all amplification mixtures without DNA sample).

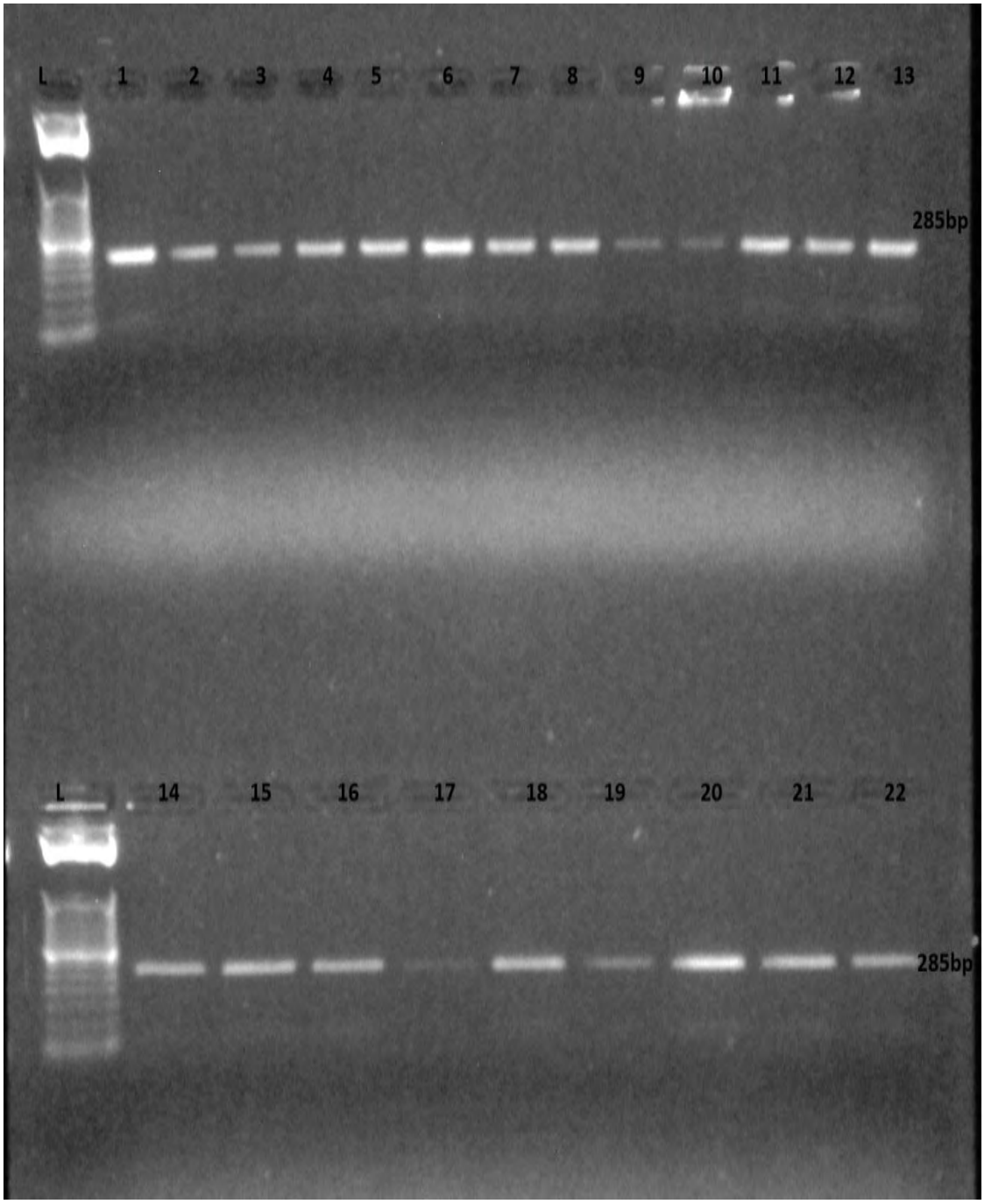

Figure 10. Electrophoretic pattern of PCR products for Mediterranean type mutation (L=ladder, Lane 2-14 and 15-24 are DNA samples).

In this study both African A- and Mediterranean type mutation which cause mild and severe deficiency are not observed. The pattern of amplification and cutting of the 211bp fragment of normal gene for African type (A-) and 285bp fragment of normal gene for Mediterranean mutation was indicated in Fig. 11 and 12 respectively.

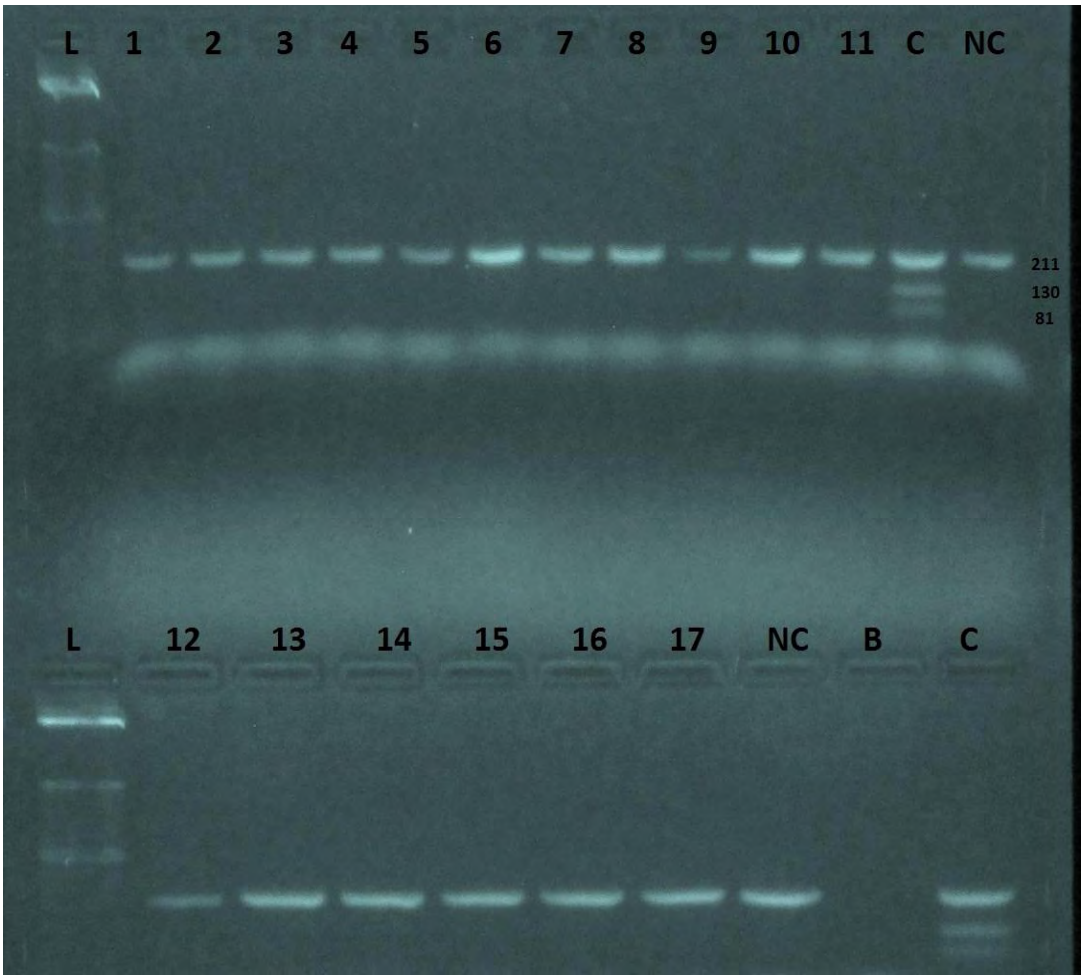

Figure 11. Electrophoretic pattern of PCR products digested with *NlaIII* restriction enzyme (L= ladder, Lane 2-12 negative samples for African A- type mutation, C= positive controls for African A- type mutation (heterozygote female), NC= negative control for African A- type mutation and B= blank).

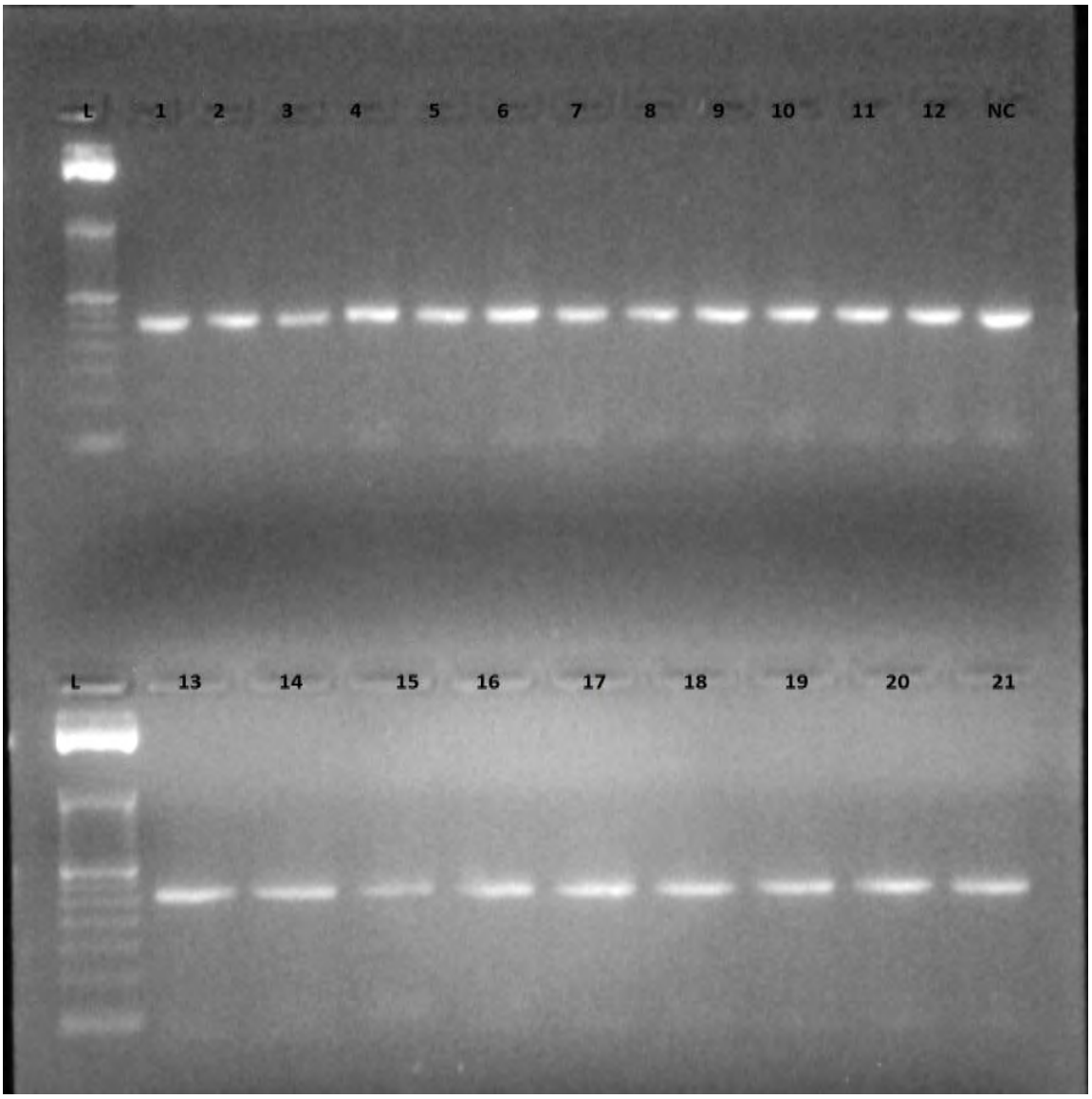

Figure 12. Electrophoretic pattern of PCR products digested with *MboII* restriction enzyme (L= ladder, Lane 2-13 and 16-24 are negative DNA samples for Mediterranean type mutation and NC=negative control for Mediterranean type mutation).

5. DISCUSSION

The molecular defects of the G6PD gene have been characterized in 620 individuals (326 female and 294 male) in Oromia, Gambella and Benishangul Gumuz regions. The frequency of G6PD A type mutation is higher in female (47) than in male (15). Three types of variants were characterized and out of the three variants, G6PD African A type variant was found to be the most prevalent type representing 10% (62/620) of the cases in the combined samples Oromia, Gambella and Benishangul Gumuz regions.

Numerous mutations in G6PD gene cause enzyme deficiency in red blood cells (Phompradit et al., 2011). According to a pervious study, 7.3% G6PD deficiency has been reported in Gambella using CareStart™ G6PDd screening test (Access Bio, New Jersey, USA) (Tsegaye et al., 2014), but in this study the variant that cause deficiency was not identified, and this could be attributed to the method they used. The frequency of G6PD A allele was 10.5% in Gambella which was grouped as non deficient variant (Abdoul Karim et al., 2014), because G6PD A type have 60-90% normal activity compared to the wild type (Stanton 2012) and the deficiency reported by Tsegaye et al., 2014 was may be due to other variants.

The present study showed that the distribution of G6PD A type variant in Oromia, Benishangul Gumuz and Gambella regions was 10%. According to World Health Organization about 2.9% of the world populations are genetically G6PD-deficient and G6PD deficiency in Ethiopia was estimated to range 0.5-2.9% using semi-quantitative assessment screening methods (WHO Working Group 1989). Therefore the deficiency reported in Ethiopia by WHO may be caused by Other deficient variants associated with mutation A376G such as : A542T (exon 6), G680T (exon 7) and T968C (exon 9) in the G6PD gene (Abdoul Karim et al., 2014) or other uncharacterized mutations at DNA levels in this study.

In the areas where mild to severe G6PD deficiency is highly prevalent, it is important to consider G6PD deficiency during treatment of malaria or other diseases. For example, medical practitioners should be aware about G6PD status of patients when prescribing drugs, especially anti-malarial drugs, and avoid prescribing antioxidant drugs to peoples with G6PD deficiency (Nakhaee, et al., 2012). However, this study revealed no mutation that can cause mild to severe G6PD deficiency.

In Africa, a variant form G6PD A, occurs with a gene frequency of approximately 0.2 (Beutler et al., 1989), which is higher when compared with 0.1 frequency obtained malaria endemic areas in Ethiopia. The gene frequency also varies between regions. G6PD A has normal activity but a more rapid electrophoretic mobility than G6PD B. G6PD A was recently showed a mutation at nucleotide 376, causing aspartic acid (GAU/GAC codons) for asparagine (AAU/AAC codons) that characterizes the enzyme and causes its rapid electrophoretic mobility (Beutler, et al., 1989).

G6PD A (376A-G) was found to have a widespread distribution, accounting for allele frequency of 0.0652 in Oromia, 0.063 in Benishangul Gumuz and 0.074 in Gambella regions. Moreover, a high proportion of the mutant allele of G6PD-D is harboured by heterozygous females (0.645 or 65%), others are homozygous females (14%) and hemizygous males (21%) among deficient individuals. The prevalence of G6PD African A type mutation was higher among female (79%) compared to male (21.0%) of deficient subjects. Prevalence of African A (376A-G) variant was higher when compared with G6PD Africa A- and Mediterranean type variant (0.00%) in the study areas.

The G6PD 376A→G variant had very high prevalence among sub-Saharan African populations, affects over 10% of individuals surveyed; further, in a subset of ten out of 16 surveys, its frequency reached 25-50% (Howes et al., 2013) which is higher than in Ethiopian malaria endemic areas which is approximately 10%. Low prevalence of G6PD A (376A→G) was reported from two surveys (n = 46 and n= 55) from northern Sudan, with <5% frequency (Howes et al., 2013b), which was lower than the current study in the Ethiopia population.

G6PD A- which classified as *class III* variant causes mild hemolysis when deficient individuals exposed to oxidative agents like antimarial drugs. In this study the prevalence of the G6PD A- genotype is null. In other parts of Africa, for example in Uganda the prevalence was reported to be 20.4%. This rate is within the range of 15-30% that has been found elsewhere in sub-Saharan Africa (Bwayo et al., 2014). Contrary to the result of present study in Ethiopia, in other African countries like Comoros Islands 3.6% (82.4% A− (376G, 202A), Mali (Dogon of Bandiagara) 16.5%, Senegal (Niakhar region/Sereer population) 16.3 % (81.4% A− (376G, 968C) 10.0% A− (376G/202A) 7.1% A− (376G/542T) (Beutler, et al., 2007) and in Burkina Faso (9.5%) the G6PD A- (A376G/G202A) is the most common variant in Sub-Saharan African countries (Abdoul Karim et al., 2014).

In this study, we investigated only one combination of mutations G6PD A- (A376G/G202A) out of four combinations (202A/376G; 376G/542T; 376G/680T and 376G/968T) responsible for G6PD deficiency in individuals living in a malaria endemic area (Abdoul Karim et al., 2014). This may be the reason why the present study could not detect Africa A- mutation in malaria endemic areas in Ethiopia. The absence of G6PD A- (A376G/G202A) mutation was also reported in north Sudan (Howes et al., 2013b).

The term ‘Mediterranean’ was used to describe the 563 T→C mutation in exon 6 of the human G6PD gene (changing a serine to a phenylalanine residue at position 188 of the protein product), reflecting the first description of this variant (in countries G6PD deficiency) in many Middle Eastern countries. This variant has also been documented in Indian subcontinent and Far East countries like China, Malaysia and Singapore (Jamornthanyawat et al., 2014).This mutation is the most common mutation in Asian Arab countries and Egypt, with frequencies ranging from as low as 53.6% in Jordan to as higher as 91.2% in Bahrain. It is the second most common variant in some African Arab countries like Algeria and Tunisia with frequencies of 23% and 11.4%, respectively (Al-Musawi et al., 2012), but the present study showed the absence of Mediterranean type mutation in malaria endemic areas in Oromia, Benishangul Gumuz and Gambella regions in Ethiopia.

The absence of G6PD Mediterranean mutation (563 T→C) in individuals among the three screened regions in Ethiopia may be attributed to the fact that it did not arise here or not yet introduced. Possibly this mutation arise somewhere in the Mediterranean basin and spread to Middle East and North Africa by extensive trade routes and colonization by the Greeks in the first millennium BC. The pattern of distribution of the Mediterranean mutation makes it quite conceivable that it may have spread through the army of Alexander the Great who occupied the Middle East and North Africa and went as far east as India (Al-Allawi et al., 2010).

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

It was concluded from the present study that G6PD A variant (376 A-G) has a wide prevalence in the studied population living in Oromia, Benishangul Gumuz and Gambella malaria endemic areas. However, the G6PD A- variant (due to simultaneous existence of 202 G→A mutation and (376 A→G mutations) and Mediterranean variant (563 T→C) were not detected. In addition, G6PD deficient persons may bear other type of G6PD A- mutation caused by 376G/542T; 376G/680T and 376G/968T) or as yet uncharacterized at the molecular level responsible for G6PD deficiency in individuals living in a malaria endemic areas. The G6PD variant reported in this study (G6PD A) can contribute to the evidence-based use of antimalarial drugs like primaquine for treatment of malaria infection in Ethiopia.

6.2. Recommendation

Future studies should be conducted on detection of G6PD deficiency, which are based on the assessment of the NADPH production capacity of G6PD. This will reduce the large use of reagents extended to analyze the DNA of non-deficient individuals. In addition variants not included in this study, like 376G/542T; 376G/680T and 376G/968T) and other that cause G6PD deficiency must be studied in the futute.

Limitation of the Study

One limitation of the present study is the use of single screening method (molecular technique) in which, one primer can detect only one type of variant. Thus only one type of Africa variant was studied which causes mild/moderate types of G6PD deficiency. Another limitation of the study was lack of the positive control sample for the Mediterranean type mutation.

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8. ANNEXES

Annex I. Preparation of the Gel

1. Weigh out the appropriate mass of agarose (2gm) into an Erlenmeyer flask. Agarose gels are prepared using a w/v percentage solution. The concentration of agarose in a gel will depend on the sizes of the DNA fragments to be separated. The volume of the buffer should not be greater than 1/3 of the capacity of the flask.
2. Add running buffer to the agarose-containing flask. Swirl to mix. The most common gel running buffers in this study is TBE (45 mM Tris-borate, 1 mM EDTA).
3. Melt the agarose/buffer mixture. This is most commonly done by heating in a microwave, but can also be done over a Bunsen flame. At 30 s intervals, remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved.
4. Add 10 μg/ml Gelred nucleic acid stain for 15-30 min.
5. Allow the agarose to cool either on the benchtop or by incubation in a 65 °C water bath Failure to do so will warp the gel tray.
6. Place the gel tray into the casting apparatus. Alternatively, one may also tape the open edges of a gel tray to create a mold. Place an appropriate comb into the gel mold to create the wells.
7. Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box alternatively, the gel can also be wrapped in plastic wrap and stored at 4 °C until use.

Annex II. Setting up of Gel Apparatus and Separation of DNA Fragments

1. Add loading dye to the DNA samples to be separated. Gel loading dye is typically made at 6X concentration (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol). Loading dye helps to track how far your DNA sample has traveled, and also allows the sample to sink into the gel.
2. Program the power supply to desired voltage.
3. Add enough running buffer to cover the surface of the gel. It is important to use the same running buffer as the one used to prepare the gel.
4. Attach the leads of the gel box to the power supply. Turn on the power supply and verify that both gel box and power supply are working.
5. Remove the lid. Slowly and carefully load the DNA sample(s) into the gel. An appropriate DNA size marker (50bp) should always be loaded along with experimental samples.
6. Replace the lid to the gel box. The cathode (black leads) should be closer the wells than the anode (red leads). Double check that the electrodes are plugged into the correct slots in the power supply.
7. Turn on the power. Run the gel until the dye has migrated to an appropriate distance.

Annex III. Observing Separated DNA fragments

1. When electrophoresis has completed, turn off the power supply and remove the lid of the gel box.
2. Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.
3. Remove the gel from the gel tray and expose the gel to uv light. This is most commonly done using a gel documentation system. DNA bands should show up as white fluorescent bands. Take a picture of the gel.
4. Properly dispose of the gel and running buffer per institution regulations.

Annex IV. Preparation of Tris-borate-EDTA (TBE) buffer

TBE buffer can be made and stored in concentrated stocks of 5× or 10×.

Composition of 1x TBE buffer

■ 89 mM Tris (pH 7.6)

■ 89 mM boric acid

■ 2 mM EDTA Preparation of 10 x TBE stock solutions to prepare 1 liter of 10× TBE dissolve following components in 600 ml deionized water:

■ 108g Tris base

■ 55g boric acid

■ 40ml 0.5 M EDTA (pH 8.0) Adjust final volume to 1 liter with deionized water.

Preparation of 1x working solution from 10 × stock buffers

To prepare a 1x working solution from 10 × stock buffers, mix 10× stock buffer with DNAse free deionized water at 1:9 ratio.