ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF MEDICINE AND HEALTH SCIENCES

DEPARTMENT OF MEDICAL BIOCHEMISTRY

The prevalence of Glucose-6-Phosphate Dehydrogenase Deficiency among Apparently Healthy Individuals in Selected Malaria Endemic Areas from Different Agroecological Zones of Ethiopia using Phenotyping and Genotyping approaches

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the award of Master's Degree in Medical Biochemistry.

By

Getasew Shitaye

September, 2017

Addis Ababa, Ethiopia

This is to certify that the thesis prepared by Getasew Shitaye entitled: **The prevalence of Glucose-6-Phosphate Dehydrogenase Deficiency among Apparently Healthy Individuals in Selected Malaria Endemic Areas from Different Agroecological Zones of Ethiopia using Phenotyping and Genotyping approaches** and Submitted in the Partial Fulfillment of the Requirements for the Degree of Master of Science (Medical Biochemistry) complies with regulation of the university and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

Examiner Dr. Signature…….Date…….

Advisor Dr. Menakath Menon Signature…….Date…….

Advisor Dr. Endalamaw Gadisa Signature……..Date……

Advisor Mr. Fitsum Girma Signature……..Date……

Principal Investigator

Getasew Shitaye

Addis Ababa University School of Medicine

Department of Biochemistry

Email: - getashitaye@gmail.com

Mob: - 0913272345

Advisors

Dr. Menakath Menon

Email: - menakathmenon@gmail.com

Addis Ababa University School of Medicine

Department of Biochemistry

Mob: - 0923244176

Dr. Endalamaw Gadisa

Malaria & Neglected Tropical Diseases Research Team

Armauer Hansen Research Institute

Email: - endalamawgadisa@yahoo.com

Mob: - 0911868827

Mr. Fitsum Girma

Addis Ababa University, Institute Of Biotechnology

Armauer Hansen Research Institute

Email: - fitsezemicheal@gmail.com

Mob: - 0912627540

ACKNOWLEDGEMENT

Before all I raise my heart in gratitude to God almighty for all the blessings He has showered on me throughout my life.

I would like to acknowledge the Department of Biochemistry, Addis Ababa University for giving me this opportunity to perform this research. I would like to pass my deepest gratitude to Bahir Dar University for sponsoring my education and financial support for living expenditure.

I gratefully acknowledge the support by Armauer Hansen Research Institute. I would like to forward my deepest gratitude to wards my advisor Dr Menakath Menon who generously gave his time and expertise to guide me.

I wish also to express my sincere thanks and appreciation to Dr Endalamaw Gadisa for giving me a good guideline throughout numerous consultations. I also appreciate his commitment to help, valuable supervision, fruitful discussion and dedication for editing the paper from beginning to writing up of the final thesis.

I cordially thank Mr Fitsum Girma who is inspiring role model and influenced me in my scientific work through his courage and help. I am glad for he devoted his time to train me in numerous laboratory works, for his guidance and giving valuable comment from proposal development to thesis write up.

Mr Temesgen, Mr Wakwaya, Mr Getaneh, Mr Mulualem and Mr Girma have contributed for success of this paper and I acknowledge all of them.

It is my pleasure to express my heartfelt thanks to Regional, Zone and Woreda Health Bureau workers of all study areas for their cooperation to facilitate the work. I would like to convey my indebtedness to study participants for their willingness in sample collection.

My sincere thanks go to all Armauer Hansen Research Institute staffs for their hospitality, kindness and cooperation. I gratefully acknowledge Data Management and statistics unit members for helping me in data entry. Special thanks go to Mr. Tsegaye Hailu for his constructive guidance and practical support on data analysis.

I pass my warmest love to my families who always inspire and motivate me through encouragement, giving me love and support for the success in my education.

Contents

ACKNOWLEDGEMENT IV
LIST OF ACRONYMS VII
LIST OF TABLES VIII
LIST OF FIGURES IX
ABSTRACT X
1. INTRODUCTION 1
1.1 Background 1
1.2 Statement of the problem 3
1.3 Significance of the Study 4
2. LITERATURE REVIEW 5
2.1 The Genetics and Inheritance of Glucose-6-Phosphate Dehydrogenase (G6PD) 5
2.2 The Prevalence of G6PD Deficiency (G6PDd) 7
2.3 Risk Factors and Diagnosis of G6PDd 10
3. OBJECTIVE 11
3.1 General Objectives 11
3.2 Specific Objectives 11
4. METHODS AND MATERIALS 12
4.1 Study Design and population 12
Study Period 14
4.3 Population 14
4.3.1 Target Population 14
4.3.2 Study Population 14
4.3.3 Inclusion and Exclusion criteria 14
4.4 Sampling method 14
4.4.1 Sample Size Determination 14
4.4.2 Sampling Procedure 14
4.5 Variables 15
4.5.1 Dependent Variables 15
4.5.2 Independent Variables 15
4.6 Data collection 15
4.6.1 Socio demographic detail of the participants 15

4.6.2 Blood sample collection and phenotypic screening 16
4.7 Laboratory investigations 17
4.7.1 DNA extraction and PCR investigation of *P. falciparum* and *P.vivax* infection 17
4.7.2 Master Mix preparation of nPCR (Nested polymerase chain reaction) Assay 18
4.7.4 Gel preparation and electrophoresis 19
4.7.5 PCR–RFLP for genotyping of G6PD deficient variants 19
4.7.7 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis 20
4.7.8 Master Mix Preparation for Digestion of Amplified Products 20
4.7.9 Electrophoresis Running Condition 21
4.7.10 Sequencing to detect uncommon G6PD-deficient variants 21
4.8 Quality Assurance 22
4.9 Data Processing and Analysis 22
4.10. Ethical Considerations 22
4.11. Dissemination and Utilization of Results 22
5. Results 23
5.1 socio-demographic characteristics 23
5.2 Malaria infection prevalence by Rapid Diagnostic Test (RDT) kit and PCR 24
5.3 Risk factors associated with malaria prevalence 27
5.4 Glucose-6-phosphate Dehydrogenase deficiency phenotype prevalence 29
5.5 Detection of *G6PD* gene variants in the deficient subjects identified by the phenotypic method 31
5.6 G6PD gene sequencing 34
5.6.1 Identification of markers in linkage and haplotype in the population 39
6. Discussion 41
7. Conclusion 45
8. Limitation of the study 46
9. Recommendation 47
10. Reference 48

LIST OF ACRONYMS

DBS	Dried Blood Spot
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
G6PD	Glucose -6-Phosphate Dehydrogenase
G6PDd	Glucose-6-Phosphate Dehydrogenase Deficiency
nPCR	Nested Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SOP	Standard Operating Procedure
TBE	Tris Borate Ethidium
UV	Ultra Violet
SNP	Single Nucleotide Polymorphism
LD	Linkage Disequilibrium
RDT	Rapid Diagnostic Test
PQ	Primaquine
HF	Haplotype Frequency
WHO	World Health Organization

LIST OF TABLES

Table 1: Classification of G6PD variants, as per WHO grading 7
Table 2: Sequences of the primer pairs used, amplification conditions for (N1) genus level and (N2) species level amplification and the expected amplicon size. 18
Table 3: Master Mix Preparation for RFLP 20
Table 4: PCR/RFLP analysed SNPs of G6PD variants and interpretation of the result. 21
Table 5: Socio-demographic characteristics of study participants, Ethiopia, 2017 23
Table 6: Percentage distribution of malaria infection by age and study sites, Ethiopia, 2017 25
Table 7: Multivariate and bivariate logistic regression analysis of explanatory variables and malaria infection using RDT among different study sites, Ethiopia, 2017. 28
Table 8: Demographic characteristics of participants with G6PDd prevalence and number of malaria episodes across different study areas, Ethiopia, 2017 30
Table 9: G6PD single nucleotide polymorphisms (SNPs) identified from sequencing 35
Table 10:G6PD Mutations Identified from all study sites, Ethiopia, 2017 36
Table 11: Association of the polymorphic SNPs and the malaria infection status as detected by nPCR, Ethiopia, 2017 37
Table 12: Polymorphic SNPs associated with asymptomatic malaria infection as detected by nPCR 38
Table 13: Haplotype frequency (HF) per each SNP blocks the G6PD gene, Ethiopia, 2017. 40

LIST OF FIGURES

Figure 1: Crystallographic structure of human wild type G6PD enzyme (adapted from Saúl Gómez-Manzo, 2016..... 5
Figure 2: Diversity of mutations in the G6PD gene and enzyme (adapted from Howes et al., 2013a) 6
Figure 3: National allele frequency of G6PD deficiency (adapted from Howes et al., 2013a) 9
Figure 4: Geographic Locations of the community or school based G6PD cross-sectional survey, July 2016 to August 2017, Ethiopia..... 13
Figure 5: Representative gel picture for *P.vivax* infection detection using nPCR..... 26
Figure 6: Representative gel picture for *P.falciparum* infection detection using nPCR. 26
Figure 7: Polymerase Chain Reaction Product for 376 A^+ Mutation 31
Figure 8: RFLP for PCR product of 376 A^+ Mutation 32
Figure 9: Polymerase Chain Reaction Product for C563T Mutation..... 32
Figure 10: RFLP for PCR product of C563T Mutation 33
Figure 11: Linkage disequilibrium plot for the whole population..... 39

ABSTRACT

Background: - Glucose-6-phosphate dehydrogenase deficiency (G6PDd) is common in malaria endemic regions that hinder the use of 8-aminoquinoline drugs as a radical cure for malaria due to the risk of inducing hemolytic anemia.

Objective: - To investigate G6PDd among apparently healthy individuals in selected malaria endemic areas from different agroecological zones of Ethiopia.

Methods: - a community based cross sectional survey involving 1609 individuals was done using genotypic and phenotypic analysis. CareStart™ Rapid Diagnostic kit (RDT) was used to screen for G6PD enzyme activity. Sequencing and polymerase chain reaction based restriction fragment length polymorphism were done for further confirmation for all phenotypically detected enzymatic deficiencies and screened representative samples from those which tested phenotypically normal. Dried Blood Spot was collected for molecular analysis. Phenotypically deficient individuals were genotyped for the mutations, G202A, A376G and C563T. *Plasmodium* blood-stage parasitaemia detection was performed using the CareStart™ Malaria Ag PLDH/HRP2 and nested Polymerase Chain Reaction.

Results: - G6PDd detected using the phenotypic approach was less (22/1609, 1.4%) than the genotypic approach (31/222, 14%). Of the 22 G6PDd individuals detected by CareStart™ RDT, 13 (1.50%) males and 9 (1.21%) females, 16/400 (4.00%), 4/484 (0.8%) and 2/401 (0.5%) were from Gambella, Oromiya and Benishangule Gumuz respectively. Moreover, the G6PDd phenotypic prevalence was significantly higher 6.50 % (13/200) in the Agnuwak ethnic groups (x^2 =47.3431 and P = 0.001). Of the 31 individuals found to be G6PDd by sequencing, 29% (9) hemizygous males, 16.13% (5) homozygous females and 54.84% (17) heterozygous females were found for $376A^+$ mutation. The highest asymptomatic malaria infection detected with RDT was *P. falciparum.*

Conclusion: - This study found high Genetic diversity (14%) across the G6PD gene in the study population. As the use of currently available radical cures (gametocidal drugs) against plasmodium are known to induce hemolytic anemia, further studies in larger groups needs to be done. In this study the limited number (222/1609) of samples sequenced from all study sites resulted in higher number of G6PDd individuals. .

Keywords: - G6PD, G6PDd, Haemolytic anaemia, DNA sequencing, Haplotype, Gene mutation, Malaria, Ethiopia.

1. INTRODUCTION

1.1 Background

Glucose-6-Phosphate Dehydrogenase (G6PD) is an X-linked cytosolic enzyme that is present in all life forms (Kumar *et al*., 2016). The human G6PD is located near the telomeric region of X-chromosome (band Xq28) (Minucci *et al*., 2009). It is a ubiquitously expressed regulatory enzyme that has a full sequence length of 18.5 Kb encoding a 59 KDa protein with 514 amino acids. It has key roles in the pentose phosphate pathway to produce nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate. NADPH plays a role in maintaining adequate reducing environment in cells which is essential in red blood cells (RBCs) function (Stanton, 2012)**.** The G6PD gene has many mutant alleles which entail a decrease in enzyme activity; expressing the G6PD deficient (G6PDd) phenotype (Shah *et al*., 2014).

In human, G6PDd is the most common enzyme defect (Ouattara *et al*., 2014) that affect estimated 400 million people worldwide (Luzzatto *et al*., 2016). More than 200 different mutations or combinations of mutations that result in over 400 G6PDd variants responsible for gradients of clinical conditions have been identified. The G6PDd variants might remain asymptomatic or result in neonatal jaundice and acute hemolytic anemia induced by medications, infection, or ingestion of fava beans due to oxidative stress on RBCs. The common G6PDd among those G6PD variant; the three variants that occur > 0.1% frequencies in sub-Saharan Africa are the wild type G6PD*B, G6PD*A (a non-deficient variant), and G6PD*A- (the deficient variant) (Ouattara et al., 2014, Shah *et al*., 2014, Carter *et al*., 2011a). Also , G6PD*A- variants with substitutions at G542T, G680T or T968C were also identified (Carter et al., 2011b, Howes *et al*., 2013b).

As G6PD gene is located on X-chromosome deficient variants are expressed more commonly in males than in heterozygous females (Ashley *et al*., 2014a). The distribution of G6PDd overlaps with malaria endemicity. This sympatric occurs has led to the speculation that malaria infection might have a selection pressure that favored the maintenance of G6PDd trait (Domingo *et al*., 2013a).

A model-based geostatistical mapping study showed that G6PDd is prevalent across malaria-endemic countries (Uyoga *et al*., 2015). Also, there is evidence that G6PD deficient individuals may be protected against severe malaria (Greene, 1993).

However, recent studies are opposing previous observations; increasing levels of G6PDd associate with decreasing risk of cerebral malaria, but with increased risk of severe malarial

anemia (Clarke *et al*., 2017). There are also data that G6PDd is a highly variable disorder, in terms of spatial heterogeneity in prevalence and molecular variants, as well as in its interactions with the spatial distribution of *Plasmodium vivax*. Unfortunately the only drug licensed for the radical cure and relapse prevention of *P. vivax* is primaquine (Bosman and Cunningham, 2013). But, Primaquine administration in G6PD deficient individuals is associated with a dose-dependent risk of 8-aminoquinolines induced life threatening hemolytic anemia (Ashley *et al*., 2014b, Domingo *et al*., 2013b, Gonçalves *et al*., 2016b) and acute renal failure (Peters and Van Noorden, 2009). WHO considers a single low dose (0.25 mg/kg) of primaquine to be safe for individuals with G6PD-deficiency (WHO, 2015), as implied by their recommendation to use primaquine as a P. falciparum gametocytocide. However, the 14-day course of primaquine (0.25–0.5 mg/kg per day for 14 days) treatment required for the radical cure of P. vivax malaria is considered to be unsafe for G6PD-deficient (G6PDd) individuals (Chen *et al*., 2015b).

The global move to control and eventually eliminate malaria has recorded huge success in the last decades. Yet, malaria continued to be a public health concern with 429, 000 reported associated deaths in 2015 (WHO world malaria report, 2015), most of whom were African and many of them were potentially preventable had prompt diagnosis and treatment were put in place. Drugs remain to be the corner stone in the fight against malaria; radical cure being the ambition especially in the elimination phase; thus understanding factors, like G6PDd that limit deployment of such drugs is timely and important.

1.2 Statement of the problem

G6PD deficiency is prevalent in Africa, Asia, Southeast Asia and parts of South America, where malaria is or has been endemic. Its prevalence is especially high in sub-Saharan Africa and Southeast Asia associated with the high malaria endemicity (Bancone *et al*., 2014).

Most of the tens of millions of clinical attacks caused by *P.vivax* each year likely originate from dormant liver forms called hypnozoites, hypnozoites of *P.ovale* as well as by *P.falciparum* infections via gametocyte development and further parasite transmission to mosquitoes (Baird, 2015b).

Though, single low dose primaquine is recommended by the WHO and is being incorporated into country policies, its use as a P.falciparum gametocytocide is growing, the wider use of 8-aminoquinolines for achieving radical cure however could induce severe haemolysis in individuals with G6PDd (von Seidlein *et al*., 2013). For this reason the diagnosis and management of G6PDd is a crucial aspect in the current phases of malaria control and elimination. The G6PD gene is highly polymorphic with almost 400 reported variants, conferring varying levels of enzyme activity. The G6PD A^- variant from Africa has been the most thoroughly studied with respect to sensitivity to primaquine (Kone *et al*., 2010). One challenging aspect to designing and interpreting clinical association studies lies in how G6PDd is defined. While many genetic association studies at G6PD implicitly assume that one nonsynonymous coding variant has both full positive and negative predictive power for G6PDd trait, this approach belies the considerable allelic heterogeneity present at the locus (Howes *et al*., 2013a, Gonçalves *et al*., 2016a), (Carter *et al*., 2011b). In parallel the diversity of G6PD phenotypes and genotypes, and our limited knowledge thereof, greatly compounds the difficulty of addressing the technical and practical limitations which this deficiency imposes on primaquine treatment for attacking the endemic malaria (Chen *et al*., 2015a).

In the Ethiopian population detailed molecular information hardly exists to indicate which variants may be responsible to G6PDd. One study conducted by Tadesse *et al*., (2015) in southwest Ethiopia documented that $G6PDA^-$(202GA) is practically absent what is expected in sub-Saharan Africa (Tadesse *et al*., 2015a). In contrast, another study by Tsegaye et al, 2014 that used phenotypic assays reported 7.3% absence of G6PD enzyme activity in southwest Ethiopia with a significant degree of variation among different ethnic groups (Tsegaye *et al*., 2014a).

Therefore, studies need to be conducted at different agroecological zones of Ethiopia on G6PD enzyme activity in combination with G6PD genotyping looking beyond the mutations determined in previous studies.

1.3 Significance of the Study

To augment the scarcity of data on G6PDd in Ethiopia, the aim of this study was to investigate G6PDd and its variants among indigenous healthy individuals in historically malaria endemic areas from different agroecological zones of Ethiopia. The study generated relevant baseline information for the national malaria control and elimination program and also for further researches that will be conducted on prevalence of G6PDd among different ethnic groups and different historically malaria endemic areas in Ethiopia.

2. LITERATURE REVIEW

2.1 The Genetics and Inheritance of Glucose-6-Phosphate Dehydrogenase (G6PD)

The G6PD is a cytosolic enzyme encoded by a housekeeping Mendelian X-linked gene. Its main function is to produce NADPH, a key electron donor in the defense against oxidizing agents and in reductive biosynthetic reactions (Monteiro *et al*., 2014). This protects the cell from oxidative attack by radicals derived from oxygen and organic compounds such as drugs and their metabolites. The role of G6PD is particularly essential in red blood cells (RBCs), which do not have mitochondria and are therefore dependent on G6PD as the only source of NADPH to relieve oxidative stress (McDonagh *et al*., 2012). In spite of its vital function, the G6PD enzyme is highly variable, both biochemically and genetically (Luzzatto and Seneca, 2014). G6PD in the active form consists of the same subunits of either dimmer or tetramer and includes tightly bound NADP (Figure 1) (Farhud and Yazdanpanah, 2008).

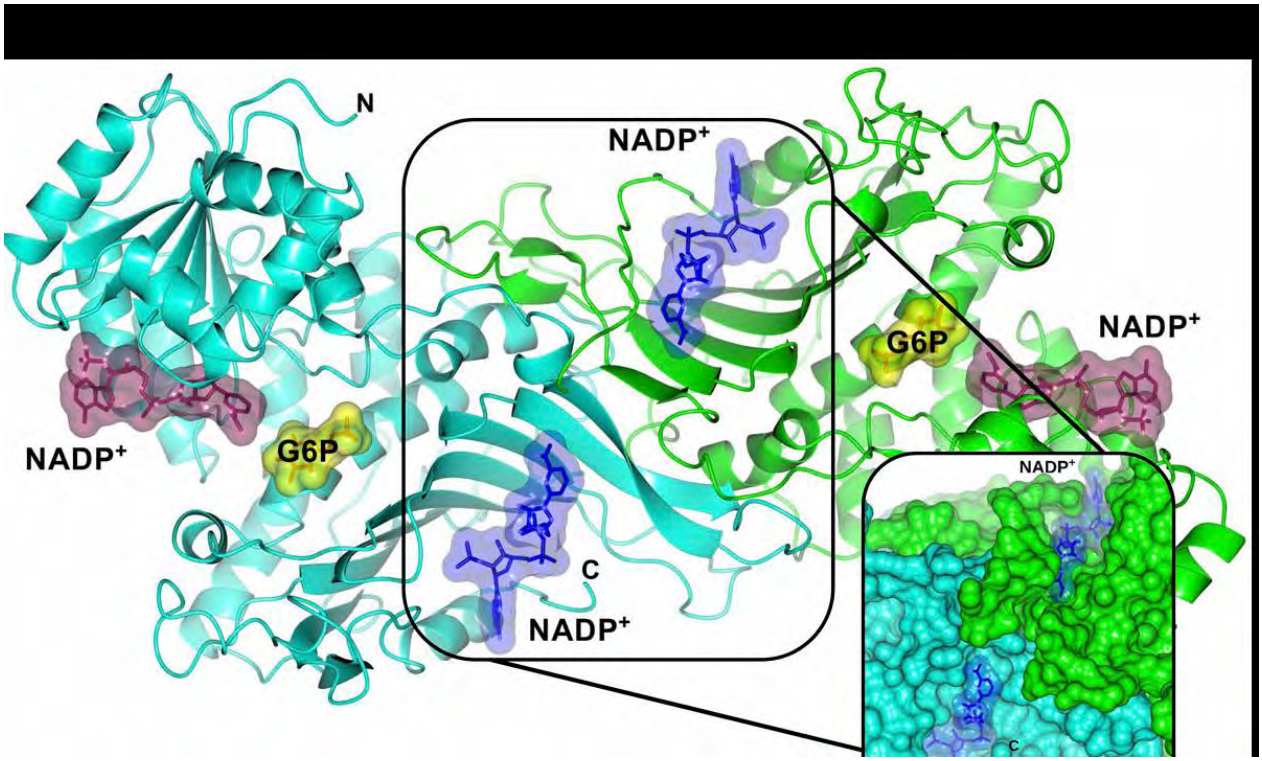

Figure 1: Crystallographic structure of human wild type G6PD enzyme (adapted from(Gómez-Manzo et al., 2017)

Currently successful mapping of the G6PD genes is available that showed its genetic diversity and variability (Figure 2).

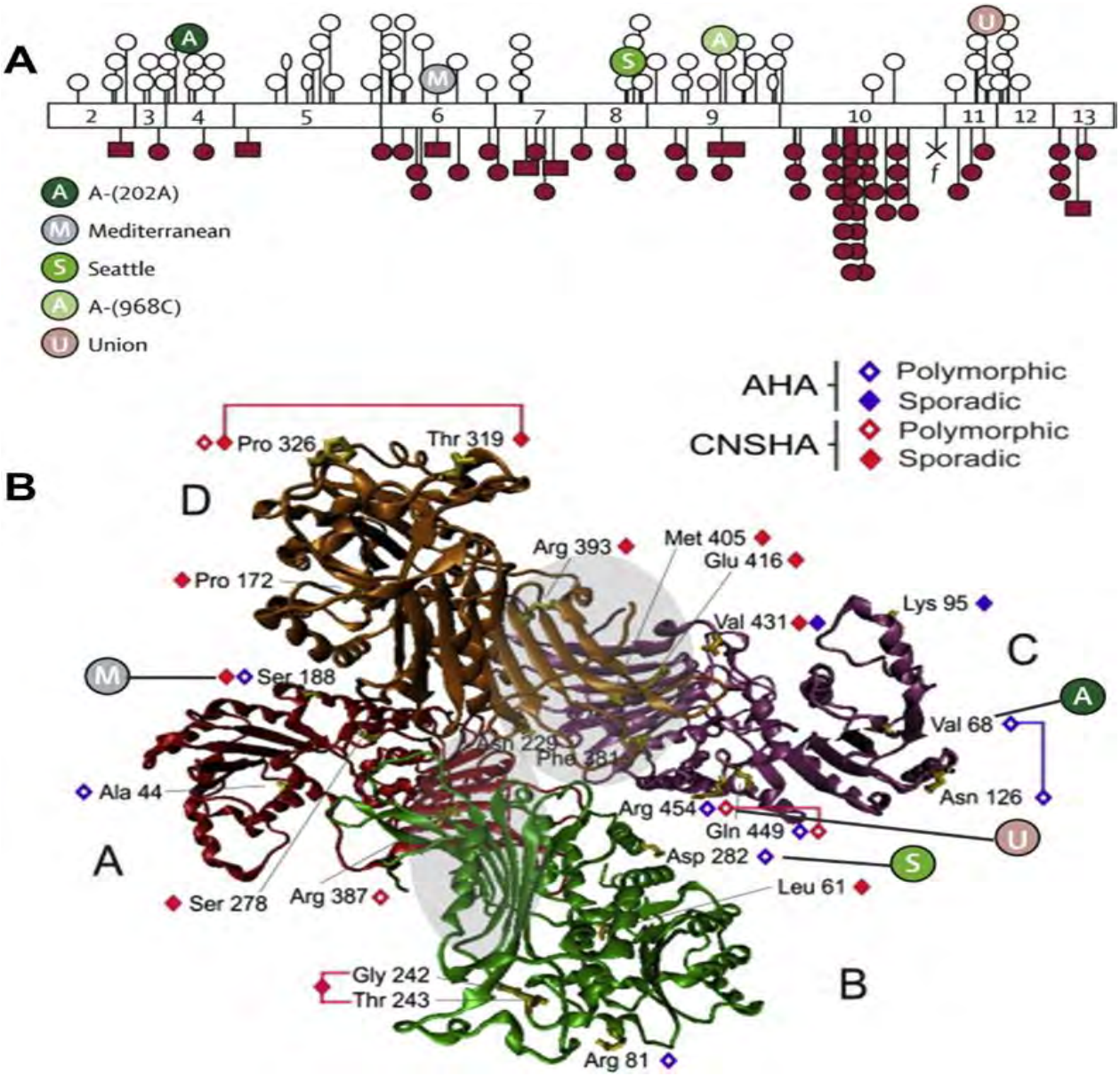

Figure 2: Diversity of mutations in the G6PD gene and enzyme (adapted from Howes et al., 2013a)

Pannel A: the distributions of the common mutations along the G6PD gene coding sequence. Open numbered boxes represent Exons; Open and Filled circles circles stand for Class II and III, and Class I variants causing mutations respectively. While the filled squares represent small deletions; the cross represents a nonsense mutation and "f" shows a splice site mutation.

Panel B shows the distribution of amino acid substitutions across the enzyme's tetrameric structure (each identical monomer subunit is labelled A–D), numbered according to the affected amino acids. The diamonds indicate polymorphic or sporadic mutations, and their colour shows the associated clinical phenotype.

The gene is one of the most highly polymorphic of the human genome with at least 186 mutations having been described (Howes *et al*., 2013a). However, many variants of G6PD are mostly produced from missense mutations with wide ranging levels of enzyme activity and associated clinical symptoms (Dallol *et al*., 2012). Males are hemizygous for the G6PD gene and can have normal gene expression or be G6PD deficient. Females, who have two copies of the G6PD gene on each X chromosome, can have normal gene expression, be heterozygous or rarely homozygous for a mutation or compound heterozygous for two mutations on the G6PD gene (Dombrowski et al., 2017, Broek et al., 2016). According to the degree of enzyme deficiency and hemolysis the World Health Organization (WHO) (Group, 1989) has categorized the mutant enzymes into five classes. The most common variants are noted in Table 1.

Table 1: Classification of G6PD variants, as per WHO grading

Class	G6PD activity	Hemolysis	Most common variants
I	<10%	Chronic	G6PD San Diego
II	<10%	Acute	G6PD Mediterranean, G6PD Chatham
III	10–60%	Acute	G6PD-A$^-$ G6PD Canton
IV	60–150%	Absent	G6PD-B, G6PD-A+
V	Increased		G6PD Verona

2.2 The Prevalence of G6PD Deficiency (G6PDd)

As per the WHO advisory committee 2013 report, the median prevalence of G6PDd varies from 32.5% in parts of sub-Saharan African and the Arabian Peninsula, to below 20% in central and Southeast Asia and 4-7% in many countries engaged in malaria elimination. The report also emphasized that the deficiency is mainly manifested in hemizygous males, with heterozygous females displaying varying levels of enzyme deficiency and homozygous deficient females being rare.

Part of the review (Monteiro *et al*., 2014) on G6PDd in Latin America assessed its varying frequencies depending on the region and ethnic group. This review documented generally low prevalence rates of G6PDd in Argentina, Bolivia, Mexico, Peru and Uruguay, but studies from Curaçao, Ecuador, Jamaica, Saint Lucia, Suriname and Trinidad, as well as some surveys carried out in areas of Brazil, Colombia and Cuba, have shown a high prevalence (>10%) of G6PDd.

The G6PD A^- (G202A) mutation was the variant most broadly distributed across Latin America that was identified in 81.1% of the deficient individuals surveyed. Also, a quantitative biochemical and genetic heterogeneity characterization study done in 2016 on the prevalence and molecular characterization of G6PDd in two *Plasmodium vivax* endemic areas of Venezuela showed predominance of the African A-202A/376G variant. Overall 24 (3.6 %) of 664 randomly recruited unrelated individuals were G6PDd by the biochemical characterization, while DNA analysis showed one or two mutated alleles in 19 of them (79.2 %). The G6PD A-202A/376G variant was the only detected in 17 (70.8 %) individuals by which 13 of them were hemizygous males and four heterozygous females. Accordingly two males carried only the 376A → G mutation and no other mutation was found in the analysed exons (Vizzi *et al*., 2016b).

Cytochemical detection of heterozygous G6PDd in 2009 documented that the frequency of mutations varies greatly among different populations. According to the study G6PD A^- was the most prevalent mutation in Africans and Afro-Americans with the class III mutation having a gene frequency of 11%. Another more severe class II deficiency, G6PD B^- (Mediterranean), was commonly found in populations living in or originating from around the Mediterranean Sea with varying frequencies that ranged from 2–20% found in Greece, Turkey, and Italy, to 70% found in Kurdish Jews (Peters and Van Noorden, 2009).

Molecular characterization of G6PDd variants in Baghdad city, Iraq was also a study conducted in 2012 among 1810 apparently healthy adult male blood donors. The result demonstrated that G6PD deficiency was detected in 109 of the 1810 screened male individuals (6.0%). From molecularly studied 101 G6PD deficient males, the Mediterranean mutation was detected in 75 cases (74.3%), G6PD Chatham in 5 cases (5.0%) and G6PD A^- in two cases (2.0%) (Al-Musawi *et al*., 2012).

A study conducted by Okebe et al, (2014) on the prevalence of G6PDd among 1,437 Gambian school children showed that the prevalence of the $202A^-$, 968 and 542 mutations were 1.8%, 2.1% and 1.0%, respectively. Higher mutation rate was seen in boys than in girls. Similarly the overall G6PDd phenotype prevalence was 6.4%; 7.8% in boys and 4.9% in girls. It was also noted that deficient phenotype was associated with reduced odds of malaria infection (Okebe *et al*., 2014a). A cross-sectional study done in north-eastern Tanzania in 2003 on the prevalence of G6PDd and haemoglobin S in high and moderate malaria transmission areas of Muheza, showed a significantly higher G6PDd prevalence in lowland compared to highland areas (Segeja *et al*., 2008). No statistically significant difference in the prevalence and incidence rates of malaria

infection among the different G6PD genotypes was found among children in Iganga district in Uganda. Among the 245 children 79.59% had the wild type G6PD A^- mutation,, 12.65% were heterozygous and 7.76% were homozygous or hemizygous. Among the males 14% were hemizygous and overall 24% of the study participants had a G6PD A-mutation (Bwayo *et al*., 2014).

A community-based cross-sectional survey conducted on submicroscopic carriage of *Plasmodium falciparum* and *Plasmodium vivax* in a low endemic area of southwest Ethiopia in 2015 did not find the G6PD African A^-(202GA) and Mediterranean (563CT) variants (Tadesse *et al*., 2015a) among 555 participants. However, a cross-sectional study among malaria suspects attending Gambella hospital, southwest Ethiopia in 2014, revealed 7.3% (33/449) G6PDd prevalence with no significant difference between the sexes. The G6PDd was higher in the native ethnic groups (Agnuwak and Nuer) compared to the 'highlanders 'or settlers (Tsegaye *et al*., 2014).

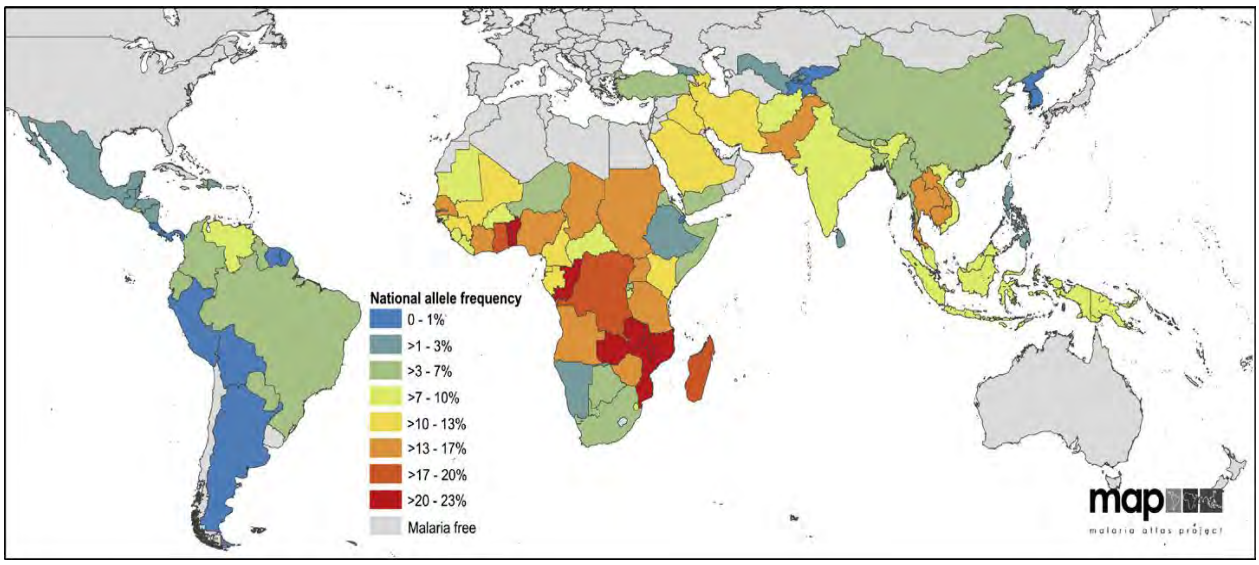

Figure 3: National allele frequency of G6PD deficiency (adapted from Howes *et al*., 2013a)

As shown in Figure 3, there is a significant association between G6PDd allele frequency polymorphism with malaria endemicity. From the map, the colored areas are malarious and there is high G6PDd allele frequency as we go down from the legend (Howes *et a*l., 2013a).

2.3 Risk Factors and Diagnosis of G6PDd

G6PDd may cause acute hemolysis or severe chronic non-spherocytic hemolytic anemia. Hemolytic anemia associated with G6PDd is usually triggered by bacterial or viral infections as well as by certain drugs. The clinical manifestation of G6PDd is closely related by ingestion of the fava bean, *Vicia faba* or certain drugs (Beutler, 1991). Neonatal jaundice is another most life and health threatening consequences of G6PD deficiency.

Following the WHO recommendations, the diagnosis of G6PDd utilizes universal tests, mainly based on the generation of NADPH from NADP (Frank, 2005). Fluorescent spot test, which is rapid, simple, sensitive, and inexpensive can be used in Countries where G6PDd is both frequent and malaria endemic, before starting treatment with antimalarial drugs, such as primaquine (Minucci *et al.*, 2009).

Another, more promising assay is Carestart™ G6PD, produced by Access Bio. It is a rapid test kit that has several advantages compared with other tests: it is easy to deploy, it is much cheaper than other G6PDd diagnostic tools, it does not have temperature range limitations, and it can be performed using capillary blood (Baird, 2015a, Chen *et al*., 2015b).

However, heterozygotes pose a particular diagnostic problem due to the Lyonization of the trait during random inactivation of one X chromosome during embryonic development (Baird *et al*., 2015). The current main diagnostic techniques used to assess G6PD status are molecular genotyping and quantitative spectrophotometry. The molecular analysis may be useful for population screening, family studies, or prenatal diagnosis. These tests are particularly important for the analysis of G6PD heterozygous females (Brito *et al*., 2016).

Molecular genotyping requires G6PDB (Glucose-6-Phosphate Dehydrogenase) full gene sequencing. However, Variants of uncertain (VUS) significance may be identified and if a VUS is identified, a WHO Class will not be assigned and enzyme studies are recommended.

3. OBJECTIVE

3.1 General Objectives

- To investigate the prevalence of G6PDd using both phenotyping and genotyping approaches among apparently healthy individuals in selected historically malaria endemic areas from different agro ecological zones of Ethiopia.

3.2 Specific Objectives

- To investigate the prevalence of G6PDd with phenotypic assay
- To assess the frequency of G6PDd genotypically targeting the most common variants in phenotypically negative individuals
- To determine the genetic variants of G6PD among study population
- To investigate novel mutations that associate with enzymatic deficiency by sequencing
- To assess parasite prevalence with RDT and PCR

4. METHODS AND MATERIALS

4.1 Study Design and population

A community and school based cross-sectional survey was conducted to investigate G6PDd both by phenotyping and genotyping approach. The study was conducted in nine woredas selected based on malaria transmission from Gambella, Benishangul-Gumuz, Oromiya and Amhara regional states.

From Gambella regional state, Abobo woreda (474 masl, N 7 3 . and 3 3 .37) and Lare woreda (422 masl, N . and 33 7 .7) were selected. Four kebelles: Chovokir, Tegni, Okuna and Village17 from Abobo, Kordeng 01, Kordeng 02, Kurgegn and Kutogn Kebelles from Lare Woreda were included.

From Benishangule Gumuz regional state Menge and Maokomo woredas were selected. From Menge (67 masl, N . and 3 7.) , four kebelles keshaf (3 7 masl, N . 7 and 3 . 6) Banieshegol (6 masl, N 7 33.3 and 3 33. 6) Belmeghua (1135masl, N . and 3 7 .) and kuduyu which has nearly similar elevation with Belmeghua were included. From Maokomo special woreda (1807masl, N .6 a nd 3 6 . 6) Ta a (7 m asl, N 7. a nd 3 7 . 7) Tulu (6 masl, N 7 33. 3 and 3 3. 3) e amesera (masl, N 3 6. 3 and 3 6.736) and Gure (7 masl, N .6 and 3 6 . 6) kebelles were included.

From romiya regional state, Gomma and Adama woredas were selected. From Gomma (masl, N 7 3.3 and 36 3 .), Cocee (masl, N 7 3.3 and 36 3 .) immu Shayee (76 masl, N 7 . and 36 .) Chedero suse (masl, N 7 3. 6 and 36 37. 6) and immu sapa (67 masl, N 7 . and 36 .6) kebelles were included. From Adama, Bato Degaga kebelle (masl, N 6 7 and 3 33)was included.

n Amhara regional state school based sampling was followed. From awi woreda (awi general primary school (masl, N 33 . and 36 .) , North Achefer woreda (Ahuri primary school (masl, N ° ′ .7″ and 36° 6 ′3. ″)) and Bahir Dar Zuria woreda (Andassa primary school (73 masl, N °3 ′ . ″ and °37° ′7. ″)) were included.

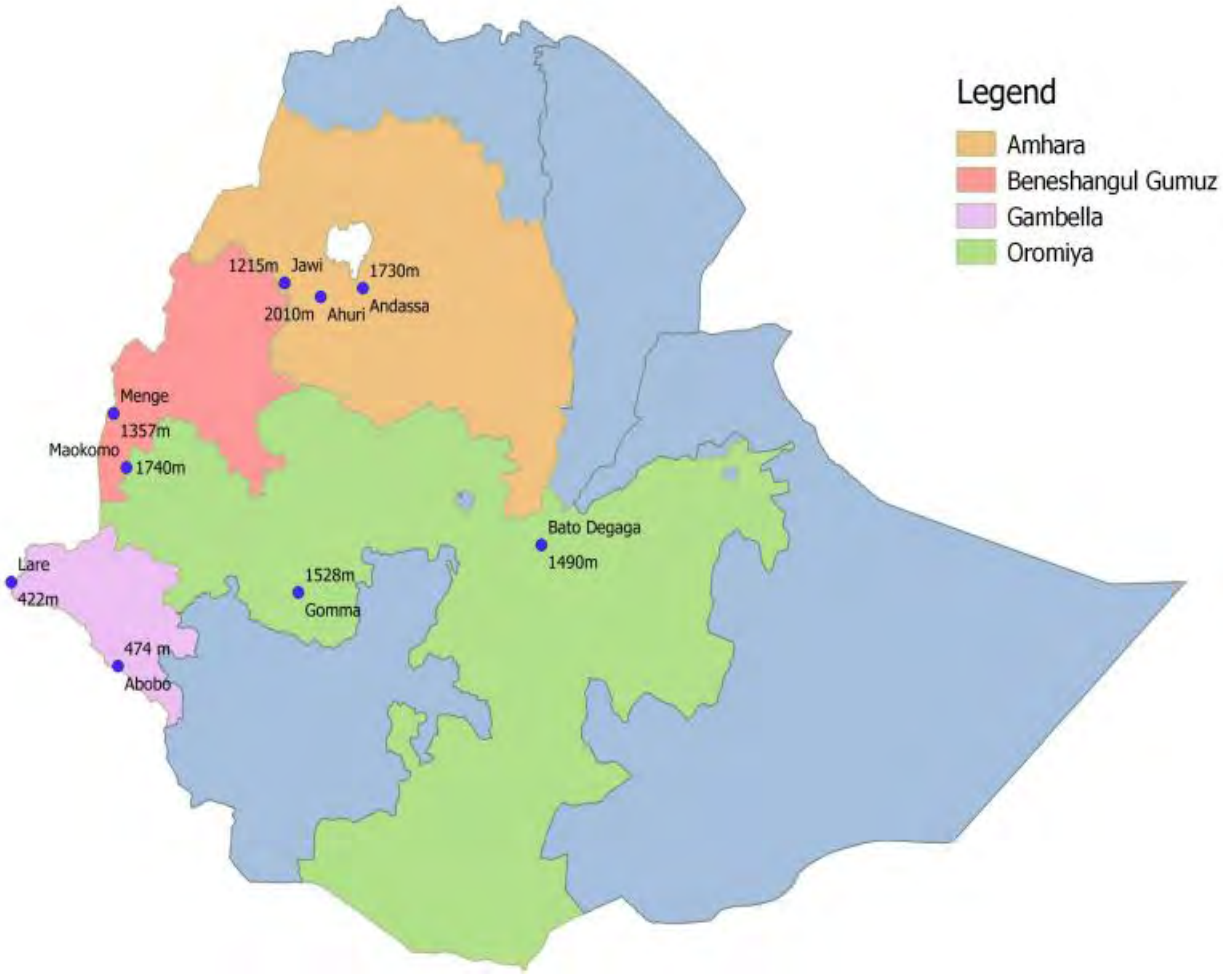

Figure 4: Geographic Locations of the community or school based G6PD cross-sectional survey, July 2016 to August 2017, Ethiopia.

Study Period

The study was conducted from July 2016 to August 2017

4.3 Population

4.3.1 Target Population

Community members who are lived in the study sites during the survey period were targeted.

4.3.2 Study Population

Volunteering apparently healthy individuals; age 1 year and above were recruited for investigating their G6PD status.

4.3.3 Inclusion and Exclusion criteria

Inclusion

- Permanent residents (more than 6 months) of the study area

Exclusion

- Individuals that are not permanent residents of the study site

4.4 Sampling method

4.4.1 Sample Size Determination

The minimum sample size was calculated by using single population proportion formula to determine the sample size by considering 7.3% prevalence of G6PDd (Tsegaye et al., 2014a), 95 % CI and a 5 % margin of error. $n = \frac{(z_{\alpha/})^2 (p (1-p))}{d^2}$

The minimum sample size was 105 that allow detection of G6PDd among populations with a 95% CI, and estimated prevalence of 4.6 – 10.8%. However from all sites 1609 individuals were screened for G6PD phenotypic screening.

4.4.2 Sampling Procedure

Apparently healthy individuals who volunteered to participate in the study and fulfill the inclusion criteria were included in the study by using purposive non probability sampling procedure. A maximum of three members of a family were screened in order to increase the power of the prediction since family members are genetically related. G6PDd is X-chromosome linked recessive disorder; males are mostly expected to have the phenotypic expression of the deficient phenotype and females though they have the genotype the heterozygotes look normal. Therefore, approximately balanced proportion of females and males were screened.

4.5 Variables

4.5.1 Dependent Variables

i. The prevalence of G6PDd based on genotyping and phenotyping
ii. Genetic variants of G6PD based on DNA sequencing

4.5.2 Independent Variables

i. Genetics (ethnic group)
ii. sex

4.6 Data collection

4.6.1 Socio demographic detail of the participants

A semi-structured Questionnaire was used to capture socio-demographics and other relevant information about study participants. A total of 1609 participants; 400 from Gambella, 401 from Benishangule Gumuz, 484 from Oromiya and 324 from Amhara regional states, were screened.

4.6.2 Blood sample collection and phenotypic screening

A finger prick blood sample (~300ul) was collected by professional nurses and laboratory technicians from study participants who gave informed consent. First, rapid diagnosis of Plasmodium blood-stage parasitaemia was performed using the CareStart™ Malaria Ag.PLDH/HRP2 (ACCESS BIO, INC.65 Clyde Rd. Suite A, Somerset, NJ 08873, USA). In parallel Participants were screened for the phenotypic G6PDd using the CareStart™ (R F G) (Access Bio, nc., New ersey, USA) following the manufacturer's instruction. The CareStart™ G6PDd test is composed of a strip impregnated with a G6PD substrate. The assay is colorization test based on a formazan formation using a tetrazolium compound. A normal G6PD whole blood sample produces a purple color in the reading window as it migrates along the strip. n contrast, a G6PDd whole blood sample shows no color. Briefly, µl of whole blood was added to the sample well and then µl of assay buffer to the buffer well immediately after application of blood. The results were read as deficient and normal within ten minutes. When a distinct purple color appeared in the reading window within ten minutes it was interpreted as Normal. When no color change or a very faint purple color appeared in the reading window within ten minutes a borderline result was read as deficient. When there was no blood migration or incomplete blood migration in the window it was recorded as invalid, and the test was repeated.

Dried blood spots (DBS) on WhatMann 3MM filter papers were collected for all participants for subsequent PCR test to check the presence or absence of one or more of the common mutations in the G6PD gene and *P. falciparum and P. vivax* infection.

4.7 Laboratory investigations

4.7.1 DNA extraction and PCR investigation of *P. falciparum* and *P.vivax* infection

Using systematic random sampling 20% of the total sample from each study site was selected. Therefore a total of 411 samples were used both for nested PCR based detection of *P.falciparum* and *P.vivax* infection and PCR-RFLP based genotyping of G6PD.

Genomic DNA was extracted using saponin-chelex dual extraction procedure as described previously (Baidjoe *et al*., 2013), Tadesse *et al*., 2015). Molecular biology grade Saponin (SIGMA ALDRICH CHEMIE GMBH) and Chelex-100 (BT chelex 100 Resin Biotechnology grade from Bio-Rad Laboratories, Inc.) were used. In brief; 6mM diameter DBS samples were punched using stainless steel puncher, soaked in 96% ethanol alcohol and sterilized by passing in a Bunsen burner flame between every sample. The DBS discs were put in specific layout in a 96 deep well plates and soaked with 820µl of 0.5% saponin solution, mounted and run on a shaker overnight. The next day, the plates were gently shaken to remove discs which might have stuck to the lid, centrifuged for 1 minute at 5,000 rpm in a Mega Fuge centrifuge (MEGA FUGE 16R, HERAEUS, Thermo Scientific, Germany). The supernatant was removed. Then 1 ml of cooled (+4 ^{o}C) phosphate buffered saline solution (PBS) was added to each well, shaken for 30 minutes and incubated at +4 oC for 30 minutes. After shaking to get the discs to the bottom of the well, the PBS solution was removed and dried with aspirator using new pipette tips to each wells. Subsequently, 150 µL of 6.0% chelex solution prepared with DNase/RNase free water (Promega Madison, WI USA) was added to each wells. Each time, the chelex was shacked to evenly distribute in the solution. To elute DNA from the clear DBS the discs were incubated in a water bath preset at 96 ^{0}C for 7 minutes, the incubation was repeated for additional 3 times by shacking and cooling at the same time on a shaker with ice at each interval. In the end the plates were centrifuged at maximum speed for 5 minutes in a Mega Fuge centrifuge. Eighty micro liter of eluted DNA solution from the spun plates were transferred into new storage plates with specific layout with care not to disturb the settled chelex. The eluted DNA was then spun at 5,000 rpm for 5 minutes in a Mega Fuge and stored at -20 ^{0}C until the next procedure (Appendix I). A nested polymerase chain reaction (nPCR) assay was performed to detect the presence of the small ribosomal subunit (18S) of *P. falciparum* and *P. vivax*. Pooled DNA isolates from *P. falciparum* NF54 cultures (Radboudumc, Nijmegen, The Netherlands) and *P. vivax* Malaria Reference Laboratory positive control (London School of Hygiene and Tropical Medicine, London, UK) were included on every PCR plate as positive controls, alongside a negative control (nucleic acid

free water). PCR products were visualized on 2% Agarose gel stained with ethidium bromide and results were subsequently photographed with UV-imager.

4.7.2 Master Mix preparation of nPCR (Nested polymerase chain reaction) Assay

PCR master mix was prepared in a separate room dedicated only for this purpose and it contained; 2mM $MgCl_2$, 0.25mM of each dNTPs, 0.25µM forward primer, 0.25µM reverse primer and 1Unit Taq DNA polymerase. Then 20µl of the N1 master mix was dispensed into 96 well PCR plates and taken to another room where 5 µl of DNA templates were added to make the final reaction volume to 25µl (Appendix III). The N2 PCR master mix was prepared in exactly the same way except 23µl of the master mix and 2µl of the N1 product and species specific primers pairs were used this time.

The PCR conditions (Snounou *et al*., 1993) for both the N1 and N2 reactions are summarized in Table 2. The final PCR mix was then brought to the amplification room and amplified in a Thermo-cycler (T100 Thermal cycler *BIO-RAD,* USA).

Table 2: Sequences of the primer pairs used, amplification conditions for (N1) genus level and (N2) species level amplification and the expected amplicon size.

Target	steps	Primer Sequence	PCR Cycling Condition	Amplicon size	References
Genera Plasmodium	Nested 1	' TTAAAATTGTTGCAGTTAAAACG ' CYTGTTGTTGCCTTAAACTTC	95^0C-10min [95^0C -60sec, 58^0C -60sec, 72^0C -90sec] 35X, 72^0C -10min	1200 BP	Snounou *et al*., 1993; Tadesse *et al*., 2015
P.falciparum	Nested 2	' TTAAACTGGTTTGGGAAAACCA AATATATT ' ACACAATAGACTCAATCATGACT ACCCGTC	95^0C-10min [95^0C -60sec, 58^0C -60sec, 72^0C -90sec] 30X, 72^0C -10min	205 BP	
P.vivax	Nested 2	' CGC T TCTAGCT TAATCCACATAACTGATAC ' ACTTCCAAGCCGAAGCAAAGAA AGTCCTTA	95^0C-10min [95^0C -60sec, 58^0C -60sec, 72^0C -90sec] 30X, 72^0C -10min	120 BP	

4.7.4 Gel preparation and electrophoresis

To visualize the amplified products 2% regular Agarose was used: 5 gm of Agarose dissolved in 250ml 0.5X TBE buffer, and boiled for 2 to 4 min in microwave to melt completely. The melted Agarose was cooled to ~37^{o}C by flashing the flask with tap water before µl ethidium bromide was added (1 µg/ml stock). The cooled Agarose was poured into clean gel casting tray with x 24 well gel combs. After solidified for 20 to 30 minutes, it was put into electrophoresis chamber that contain .X TB buffer. Then, the combs were removed, µl PCR product plus µl loading dye was loaded per well. For estimating the size 3µl of (µg/µ stock) DNA ladder was loaded alongside. Electrophoresis was run at 120 volt for 35-60 minutes. Finally the gel was put into Bio-Rad UV-transiluminator machine to visualize if amplifications specific to *P.falciparum* and *P.vivax* positivity considering 205 bp and 120 bp amplicon sizes respectively existed.

4.7.5 PCR–RFLP for genotyping of G6PD deficient variants

Extracted DNA samples were analysed for single nucleotide polymorphisms (SNPs) in G6PD: 202GA (rs1050828), 376AG (rs1050829) and G6PD:563CT (rs5030868). For the 202GA allele the forward primer was ’-CTGGCCAAGAAGATCTACCC-3’ and the reverse primer was ’-GAGAAAACGCAGCAGAGCACAG-3’(Fanello et al., 2008). For 376AG and 563CT alleles the forward and reverse primer pairs used were ’-CAGTACGATGATGCAGC-3’ and ’-CAGGTAGAAGAGGCGGT-3’(Vizzi et al., 2016a), and ’-TGATCCTCACTCCCCGAAGA-3’ and ’-GCTTGGCCCCACCTCAGCAC-3’ (Tadesse et al., 2015b) respectively. All primers pairs were from SIGMA-ALDRICH (Gillingham, UK). The PCR mix was prepared as shown in table 4 briefly, µl (~ µg/m) of DNA was amplified in a total reaction volume of 3 µl using the GoTaq Flexi DNA Polymerase (Promega, USA) (Appendix IV).

4.7.7 **Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis**

To detect the mutation A^- (202GA) the PCR condition was adopted from (Fanello et al., 2008). The cycling condition was adjusted at 95°C/5min for initial denaturation, 95°C/40sec for denaturation and 72°C/40sec for Extension. The annealing temperature of the first cycle of the touchdown PCR was 68.5°C, decreased by 0.5°C for the next 14 cycles and the annealing temperature for the last 23 cycles was 61°C with10 min of final extension at 72°C.

PCR cycling conditions for 376A mutation was performed at 40 total cycles as described by Vizzi et al. (2016). Briefly, 95°C/10min, 94°C/60sec, 56°C/30sec, 72°C/40sec was set for initial denaturation, denaturation, annealing and extension respectively. Finally it was extended for 7min at 72°C. Cycling condition established by Samilchuk et al., (1999) was used for the detection of Mediterranean type mutation (C563T). Accordingly within 30 total cycles all Denaturation (94°C), Annealing (55°C) and Extension (72°C) were performed at 45sec including Initial Denaturation at 94°C for 10min and Final Extension at 72°C for 7min.

4.7.8 Master Mix Preparation for Digestion of Amplified Products

The amplicons for 376AG, 202GA and 563CT target SNPs were digested at 37°C with the restriction enzyme *Fok I,* NlaIII and MboII for 3hrs, 4 hours and overnight respectively. All restriction enzymes were from NEBioLabs, USA. The reaction mix (Table 3) as per the recommendation of NEBioLabs contained PCR product, buffer restriction enzyme, and deionized water. The reaction mix was gently mixed by pipetting and centrifuged for a few seconds.

Table 3: Master Mix Preparation for RFLP

Ingredients	Stock concentration	working concentration	Volume(µ)/reaction
Cut smart buffer	10X	1	2
Fok I, NlaIII or MboII	5000U/mL	15U/mL	0.06
PCR Product	~ µg /m	~ µg /m	15
Deionized water			2.94
Total reaction volume			20

Table 4: **PCR/RFLP analysed SNPs of G6PD variants and interpretation of the result.**

Target SNPs	Exon	Amplicon Size In bp	Fragment size in bp	
			Wild Type	Mutant
202G → A	4	452	296[a] ,156	173, 123, 156
376A → G	5	90	90[b]	58,32
563C → T	6/7	230	202[c],28	104,98,28

Where: a = 296 bp from the wild type is cut into 173 and 123 when there is specific mutation

b = 90 bp does not cut from wild type but when there is specific mutation

c = 230 bp from the wild type is cut into 104 and 98 when there is specific mutation

4.7.9 Electrophoresis Running Condition

Electrophoresis was performed using 4% Metaphor Agarose (LZ 50180 Lonza Rockland, ME USA) (Appendix II) g Metaphor Agarose in m (. X TB) buffer. Then 7. µ l tB r (10mg/mL) was added to cooled agarose solution. After the gel was placed at 4°C for 20 minutes to obtain optimal resolution and gel handling characteristics, 15- µ of restriction products plus 3- µ loading buffer (µ / µ of sample) were loaded per wells, then 3µ of bp & bp DNA ladder (alternatively 1kb Ladder) were also loaded and electrophoresis was run in 0.5X TBE buffer. Finally, bands were visualized on UV transiluminator, photographed and consequently results recorded.

4.7.10 Sequencing to detect uncommon G6PD-deficient variants

Those samples that were found deficient with the enzymatic assay and a subset of 211 G6PD phenotypic normal samples from all the study sites were sequenced. Sequencing was done by a commercial firm.

4.8 Quality Assurance

Standard procedures were followed during blood sample collection. All molecular works were done after receiving hands on training at AHRI. The expiring dates of all chemicals and reagents were checked before use. SOPs were strictly followed, known positive controls, and non-template controls were included in all procedures. Results were interpreted against the controls and molecular size markers. Sequencing was done at the London school of hygiene and tropical medicine (LSHTM). Trainings were given to health professionals involved in sample collection on qualitative test analysis of CareStart™ G6PDd screening test. During sample collection sex and ethnicity of participants were cross checked and data entered on Microsoft excel.

4.9 Data Processing and Analysis

Self-administered questionnaires were checked for completeness and double-entered along with all laboratory results. Data was entered in Redcap and Statistical analysis was conducted using STATA 11 (StataCorp, TX, USA) and Graph Pad Prism 5.0 (Graph Pad Software Inc., CA, USA). Computation of Linkage Disequilibrium and haplotype analysis was done using Haploview version 4.2 Software. Statistical significance was set at a P value of <0.05.

4.10. Ethical Considerations

The study was carried out after it was approved by Addis Ababa University, School of Medicine Department of Medical Biochemistry Ethical (Ref.No.SOM/DRERC/BCH005/2009). The umbrella project under which this project was executed, obtained ethical approval from Addis Ababa University (CNSDO/264/08/16), Jimma University (RPGC/395/06), Armauer Hansen Research Institute (PO52/14), the National Research Ethics (310/109/2016) and the London School of Hygiene & Tropical Medicine (10628) Review Committees. Extracted DNA samples were sent to sequencing according to the Material transfer agreement stated under 10628 to London School of Hygiene & Tropical Medicine.

The purpose of the study was explained to each participant and sample was obtained only after each participant gave written informed consent and for individuals aged below 17 years in addition to assent form consent form was sought from their guardians.

4.11. Dissemination and Utilization of Results

In addition to this thesis, summary of the study result will be reported to Ministry of Health and concerned regional Health Bureaus.

5. Results

5.1 socio-demographic characteristics

A total of 1609 individuals, 745 females and 864 males participated for G6PDd screening. The median age of participants was 17 and Inter quartile range (2-75) at 95%CI (19.88-21.52). More than half of the respondents reported living in houses constructed with grass thatched roof, earthen floor, the floor and walls plastered with animal dung (Table 5).

Table 5: Socio-demographic characteristics of study participants, Ethiopia, 2017

Variables	Number	Percentage
Sex		
Male (Female)	864(745)	53.70 (46.30)
Age in years		
≤5	126	7.83
6-15	776	48.23
>15	707	43.94
Construction of house		
Type of Roof		
Grass thatch	822	63.43
Corrugated Iron sheet	474	36.57
Type of Wall		
Wood and mud	1,457	91.87
Mud and cement	124	7.82
Iron sheet	3	0.19
Brick or stone	2	0.13
Material of house floor		
Earthen	1,384	87.10
Cow dung plaster	76	4.78
Cemented	121	7.61
Wooden	6	0.38
Other	2	0.13
Eaves condition		
Open	215	15.23
Closed	1,129	79.96
Partially open	43	3.05
No eaves	25	1.77
Habit of Using bed net		
Yes (No)	1,133 (126)	88.45 (9.84)
Don't know	22	1.72
knowledge on causes and transmission of malaria		
Yes (No)	781(374)	67.62 (32.38)
Knowledge on preventive method of malaria		

Yes (No)	759 (409)	64.98 (35.02)

5.2 Malaria infection prevalence by Rapid Diagnostic Test (RDT) kit and PCR

Malaria infection assessment was done for1285 participants among 1609 screened for G6PD. The RDT result showed proportionally *high P.falciparum* (Pf) infection was detected; 22 %(84/400), 8.48 %(34/401), 1.19% (1/84) from Gambella (GA), Benishangule Gumuz (BG) and Bato Degaga (BD) respectively. Mixed Pf and *P.vivax* (PV) infection of 5.50% (18/400) from Gambella and 0.25% (1/401) from Benishangule Gumuz was detected. Only 0.25% (1/400) infection from Gomma (GO) was due to *P.vivax* (Table 8). For nested polymerase chain reaction detection of malaria infection 20% of samples were randomly selected from each site. With the nPCR, 30.38% (24/79), 18.52% (15/81) and 4.76% (4/84) prevalence of Pf was detected from Gambella, Benishangule Gumuz and Bato Degaga respectively. From Jawi, Ahuri, and Andassa only one *Pf* infection was detected. Relatively high percentage 14.29% (12/84) of PV infection was found from Bato Degaga followed by Benishangule Gumuz which was 7.41% (6/81) as summarized in Table 6.

The nPCR targeting the 18S ribosomal RNA was successfully used to detect the expected 120 bp and 205 bp sizes of N2 amplicons for *P.vivax and P.falciparum* respectively. Representative gel pictures of nPCR results are shown in Figure 5 and 6.

Table 6: Percentage distribution of malaria infection by age and study sites, Ethiopia, 2017

Area	Age (year)	% (n/N)	RDT			nPCR		
			Pf% (n/N)	PV%(n/N)	Mixed%(n/N)	Pf%(n/N)	PV%(n/N)	Mixed% (n/N)
GA	≤	5.5(22/400)	27.3 (6/22)	0.0	9.1 (2/22)	66.7 (2/3)	0.0	0.0
	6-15	39 (156/400)	23.7 (37/156)	0.0	7.7 (12/156)	31.3 (10/32)	0.0	6.3 (2/32)
	>15	55.5 (222/400)	20.3 (45/222)	0.0	1.8 (4/222)	27.3 (12/44)	0.0	2.3 (1/44)
Total			22.0 (88/400)	0.0	5.5 (18/400)	30.4 (24/79)	0.0	3.8 (3/79)
BG	≤	15 (60/401)	1.7 (1/60)	0.0	0.0	17.7 (3/17)	5.9 (1/17)	0.0
	6-15	37.2 (149/401)	15.4 (23/149)	0.0	0.7 (1/149)	29.6 (8/27)	11.1 (3/27)	0.0
	>15	47.9 (192/401)	5.2 (10/192)	0.0	0.0	10.81 (4/37)	5.41 (2/37)	2.7 (1/37)
Total			8.5 (34/401)	0.0	0.3 (1/401)	18.5 (15/81)	7.4 (6/81)	1.2 (1/81)
GO	≤	8.5 (34/400)	0.0	0.0	0.0	0.0(0/6)	0.0 (0/6)	0.0 (0/6)
	6-15	34.8 (139/400)	0.0	0.7 (1/139)	0.0	0.0 (0/26)	0.0 (0/26)	0.0 (0/26)
	>15	56.8 (227/400)	0.0	0.0	0.0	2.0 (1/49)	2.0 (1/49)	0.0
Total			0.0	0.3(1/400)	0.0	1.2 (1/81)	1.2 (1/81)	0.0 (0/81)
BD	≤	11.9 (10/84)	0.0 (0/10)	0.0	0.0	0.0 (0/10)	20 (2/10)	0.0
	6-15	47.6 (40/84)	0.0 (0/40)	0.0	0.0	5.0 (2/40)	17.5(7/40)	2.5 (1/40)
	>15	40.5 (34/84)	2.9(1/34)	0.0	0.0	5.9 (2/34)	8.8 (3/34)	0.0
Total			1.2 (1/84)	0.0	0.0	4.8 (4/84)	14.3 (12/84)	1.2 (1/84)

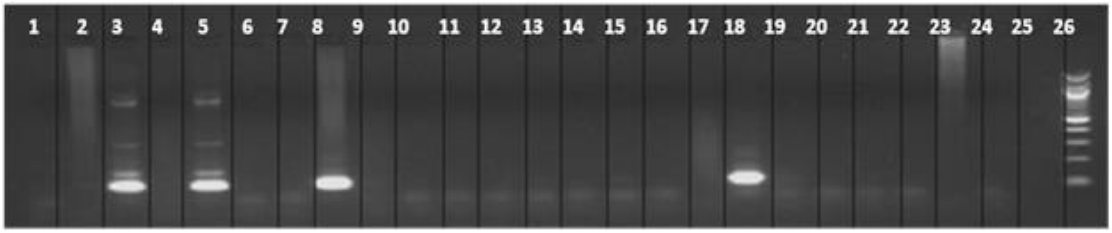

Figure 5: Representative gel picture for *P.vivax* infection detection using nPCR.

Lanes 1, 2, 4, 6, 8 = Samples, Lane 3 and 5 = positive control (known PV clinical sample), Lanes 7 and 9= non template control (nucleic acid free water), Lanes 10-22 and 24=samples, Lane 23 and lane 25=empty wells, and lane 26= 100 bp ladder.

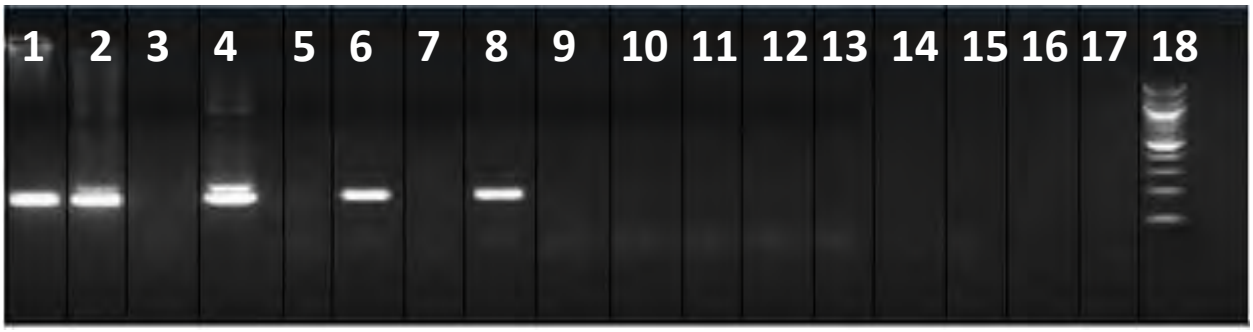

Figure 6: Representative gel picture for *P.falciparum* infection detection using nPCR.

Lanes 1, 3, 5 to 10, 12, 14 and 15 = study samples, Lanes 2 and 4= known P. falciparum positive controls, Lanes 11 and 13= non template (nucleic acid free water) negative controls, Lanes 16 and 17=empty wells and Lane 18 = 100bp ladder

.

5.3 Risk factors associated with malaria prevalence

The association between malaria infection and contributing risk factors for malaria infection was assessed using Pearson's chi-square test, multivariate and bivariate logistic regression. Risk factors that were associated in bivariate logistic regression (P≤ .) were selected and further analysed using multivariate logistic regression. Accordingly, there was no significant association (x^2=0.9775, P=0.613) between being infected and age of participant. Participants who responded that their house has electricity were at less risk for malaria incidence than those whose house didn't have (A R= . , C = . -17.6 and P=0.035). In similarly individuals who knows the cause and transmission of malaria were at less risk (AOR =2.19, CI =1.0-4.7and P=0.046). The habit of using bed net was the other factor that was significantly associated with malaria infection (AOR=4.0, CI=2-9 and P=0.001) (Table 7).

Table 7: Multivariate and bivariate logistic regression analysis of explanatory variables and malaria infection using RDT among different study sites, Ethiopia, 2017.

variables	RDT result		COR(95%CI)	AOR(95%CI)	P-value
	Positive	Negative			
Age (in Years)					
≤	9	117			
6-15	75	701			
>15	62	645			
Electricity					
Yes	3	448	1.0(reference)		
No	140	988	21.2(6.7-66.7)	4.4 (1.1-17.6)	0.035
Radio					
Yes	9	398	1.0(reference)		
No	133	1,035	5.6 (2.9-11.2)		
Television					
Yes	1	160	1.0(reference)		
No	141	1,259	17.9(2.7-128)		
Type of Roof					
Grass thatch	86	736			
Iron sheet	8	466			
Wood & mud	-	-			
Type of Wall					
Wood & mud	141	1,316	1.0(reference)		
Mud & cement	2	122	0.15(0.03-0.62)		
Iron sheet	0	3	-		
Brick or stone	0	2	-		
Material of house floor					
Earth	139	1,242	1.0(reference)		
Local dung	1	75	0.12(0.01-0.86)		
Cement	3	118	0.23(0.07-0.73)		
Wood	0	6	-		
Other	0	2	-		
Eaves condition					
Open	14	201	1.0(reference)		
Closed	83	1,046			
Partially open	10	33	4.3 (1.8-10.6)		
No eaves	3	22			
Using bed net					
Yes	62	1,073	1.0(reference)		
No	25	101	4.3(2.6-7.1)	4.0 (2-9)	0.001
Don't know	8	14	9.8 (3.9-24)	7.0 (2-20)	0.001
Cause and transmission of malaria					
Yes	62	719	1.0(reference)		
No	75	299	2.9(2.0-4.1)	2.19(1.0-4.7)	0.046
Preventive method of malaria					
Yes	62	719			
No	75	299			

5.4 Glucose-6-phosphate Dehydrogenase deficiency phenotype prevalence

CareStart™ Glucose-6-phosphate Dehydrogenase deficiency screening test was available for all 1609 participants. Total of 22 (1.37%) G6PDd phenotypes, of them 13(1.50%) males and 9(1.21%) females were found. No significant differences were found for G6PD deficiency among males and females (x^2=0.2609, p=0.609). From those 22 G6PD deficient participants two (1.63%) individuals presented *P.falciparum* infection as determined by RDT and PCR. The statistical value (P=0.998) between G6PDd and malaria infection showed as no association. There were regional differences in the prevalence of G6PDd. Among those Different ethnic groups included in each site the G6PDd phenotype prevalence was strongly associated with ethnicity (x^2 =47.3431 and P = 0.0001). The highest of all 4.00% (16/400) prevalence was found in Gambella. Among two ethnic groups included in this region 6.50 % (13/200) G6PDd were found in Agnuwak and 1.50 % (3/200) in Neur. From Oromiya region (Gomma) 0.87% (4/484) G6PD deficient individuals found and all of them were having Oromo ethnicity. Two deficient individuals among Berta ethnic groups were from Benishangule Gumuz which accounts 0.50% from 401 as shown in Table 8.

Table 8: Demographic characteristics of participants with G6PDd prevalence and number of malaria episodes across different study areas, Ethiopia, 2017

Area	Ethnicity	G6PD Result in %		Number of malaria episode		
		Normal	Deficient	Yes	No	don't know
Gambella n=400						
	Agnuwak	93.50 (187/200)	6.50 (13/200)	9.90 (38/384)	46.88 (180/384)	43.23 (166/384)
	Nuer	98.50 (197/200)	1.50 (3/200)			
Total		96.00 (384/400)	4.00 (16/400)			
Benishangule Gumuz n=401						
	Berta	99.16 (237/239)	0.84 (2/239)	30.71 (121/394)	69.29 (273/394)	0.00
	Mao	100 (118/118)	0.00			
	Komo	100 (18/18)	0.00			
	Oromo	100 (25/25)	0.00			
Total		99.50 (298/400)	0.50 (2/401)			
Oromiya n=484						
	Oromo	98.66 (369/458)	0.87 (4/458)	11.13 (52/467)	88.86 (415/467)	0.00
	Other	100 (26/26)	0.00			
Total		99.00 (399/400)	0.83 (4/484)			
Amhara n=324						
	Amhara	100.00 (324/324)	0.00	19.13 (62/324)	80.86 (262/324)	0.00

5.5 Detection of *G6PD* gene variants in the deficient subjects identified by the phenotypic method

Twenty two G6PDd phenotypes that were found by the CareStart™ G6PDd screening test were genotyped for investigation of 202GA (rs1050828), 376AG (rs1050829) and 563CT (rs5030868) gene mutations with PCR-RFLP. However, genotyping result for 202GA was failed after several attempts and finally the problem was resolved that the primer sequences were not amplify at specified target. Thus, for this target only sequencing result is presented in 5.6 below. For the two variants PCR and RFLP result presented below with figures.

All those G6PDd by the CareStart™ were amplified by targeting 90bp of the wild type A376G G6PD gene variant (fig.7). The fragments amplified were then digested for 3hrs at 37°C with the restriction enzyme Fok I (Fig.8). As a result six hemizygous males and one homozygous female were found mutated.

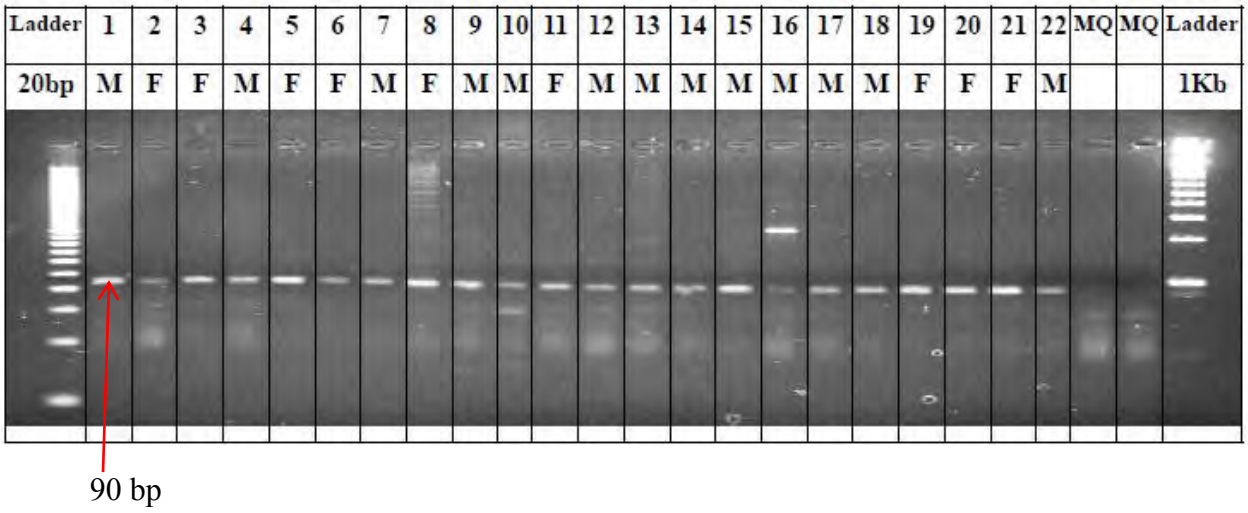

Figure 7: Polymerase Chain Reaction Product for 376 A^+ Mutation

anes “ladder” = (bp) and (K b)= DNA molecular markers, Lanes 1 to 22=study samples, and lanes “MQ” = Negative control (nucleic acid free water).

NB: the non-template control lanes “MQ” consistently produced ~60bp amplicon irrespective of the effort to optimize. We latter on learned that the two primers had a probability of ligate and the exponential nature of PCR amplification with the higher probability of self-amplification might result in such product.

5'-CTGGCCAAGAAGATCTACCC-3'
"5'-GAGAAAACGCAGCAGAGCACAG-3'". This phenomenon is vivid in figure 7, in the presence of the target sequence the primer pairs are consumed the probability of formation of this product is less.

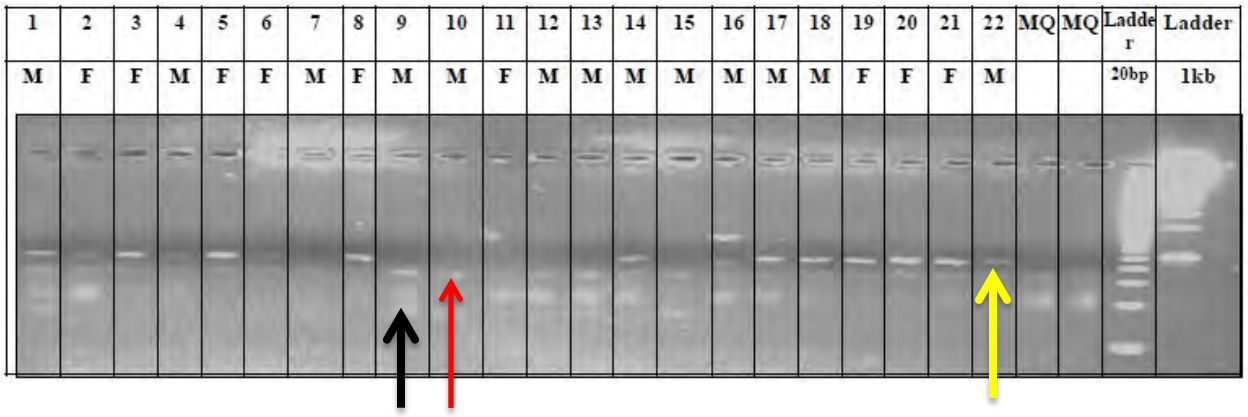

Figure 8: RFLP for PCR product of 376 A^{+} Mutation

anes to = Study samples, anes "MQ"= Non template controls and anes "ladder"= bp and 1Kb DNA molecular markers.

NB: the interference of the primer amplification did not confound or PCR-RFLP interpretation; in lanes 9, 10, and 15 there are ~58 bp (red arrow) of 32 bp (black arrow) fragments. Thus absence of the 90 bp (PCR product size) and the nonspecific primer polymerization result (yellow arrow) enabled me for the designation of mutant phenotypes.

Fragment amplification was also done to detect the Mediterranean type mutation among the phenotypically deficient samples. As showed in figure 9, the target 230 bp of wild type was amplified.

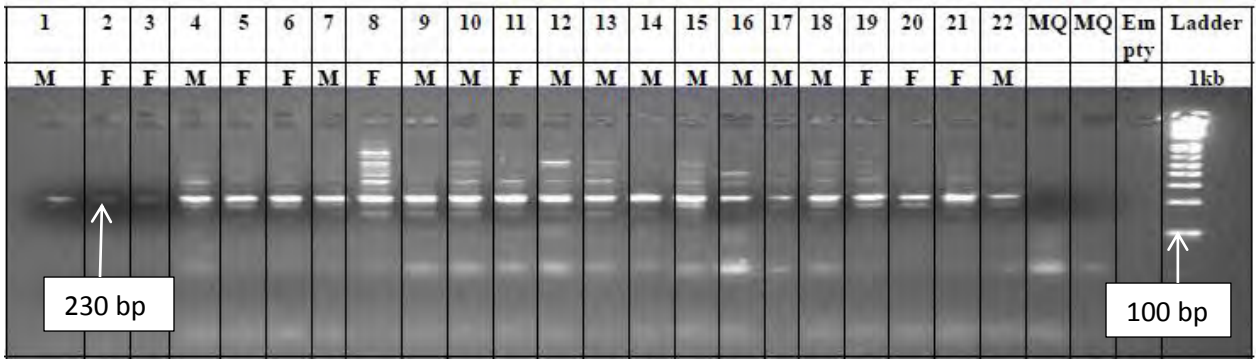

Figure 9: Polymerase Chain Reaction Product for C563T Mutation

anes to = Study samples, anes "*MQ*"= Non Template controls and adder= Kb plus DNA molecular marker.

The overnight MboII digestion at 37°C of the 563CT amplicons (Fig.10) resulted in the wild type 230 bp cut into 202 and 28 bp fragments confirming the absence of the Mediterranean gene variant among the samples that phenotypically tested deficient.

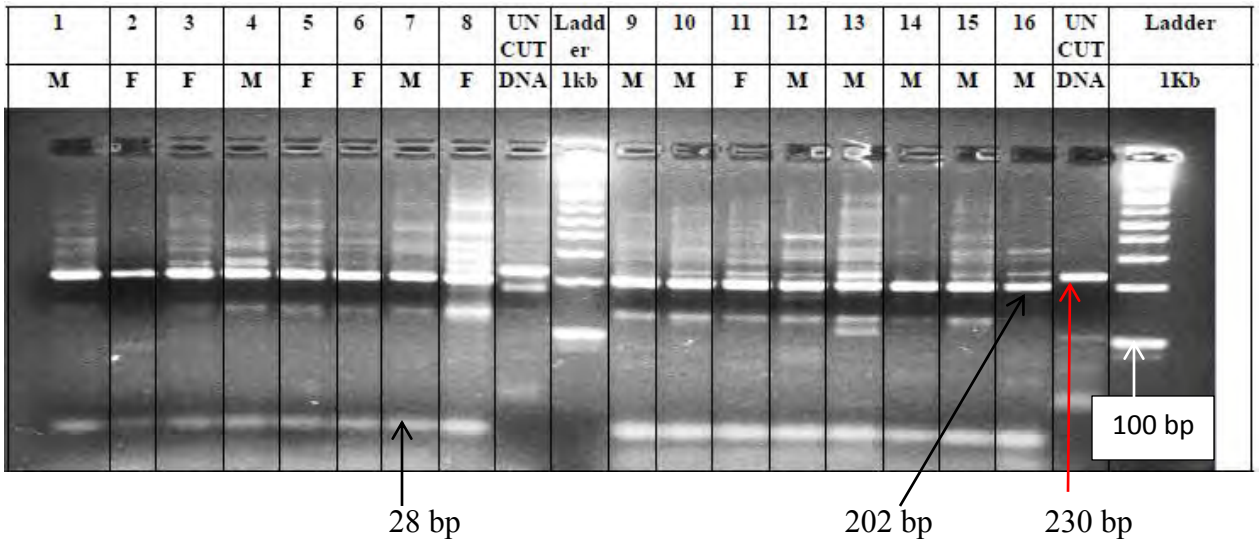

Figure 10: RFLP for PCR product of C563T Mutation

anes to 6= Study samples, lane "UN Cut"= PCR product/undigested sample and ane "ladder kb"= Kb plus DNA molecular marker.

5.6 G6PD gene sequencing

From all the study sites a subset of 222 samples of them 128 males and 94 females were selected for full G6PD gene sequencing. These samples included 11 deficient and 211 G6PD normal individuals as diagnosed by the CareStart™ Glucose-6-phosphate Dehydrogenase deficiency screening test. Overall, among 36 genetic markers sequenced 30 SNPs were identified across the G6PD genomic DNA sequence examined (Table 9). Six Genetic markers that were not polymorphic (rs5030868, rs5030869, rs76723693, rs137852318, rs137852328, rs12389569) did not list in Table 9.

As detailed in Table 10, 222 samples (128 males and 94 females) were successfully sequenced and passed quality control. Unfortunately half of the phenotypic deficient samples (7 from Gambella and 4 from Gomma) were unsuccessful and not included from the analysis. Total of 31 individuals of them 9 hemizygous males, 5 homozygous females and 17 heterozygous females were found for $376A^{+}$ mutation. Four hemizygous males and one heterozygous female were also deficient for $202A^{-}$ variant and all of them were deficient for 376. Among 11 sequenced deficient phenotypes only one hemizygous male deficient for A376G was found.

Table 9: G6PD single nucleotide polymorphisms (SNPs) identified from sequencing

X-chromosome: position [a]	Ref. SNP identifier [b]	Function	MAF [c]	Nucleotide Variation (Ref/Alt) [d]
X:154536002	rs1050828	Missense variant	0.022	G/A
X:154535277	rs1050829	Missense variant	0.113	A/G
X:154536313	rs762515	Intron 2	0.107	T/C
X:154534556	rs2515904	Intron variant	0.032	G/C
X:154533860	rs2515905	Intron variant	0.033	G/A
X:154532293	rs2071429	Intron variant	0.337	G/A
X:154532439	rs2230037	Synonymous variant	0.39	G/A
X:154527775	rs1894260		0.153	C/T
X:153761564	rs73573478	Non coding exon variant	0.065	G/A
X:154547570	rs111827785	Intron Variant	0.183	T/C
X:153764663	rs762516	Intron variant	0.032	C/T
X:153773062	rs113492957	Intron variant	0.06	C/T
X:154611562	rs2004651	Synonymous variant	0.22	T/G
X:154607918	rs60030796	Downstream gene variant	0.069	A/G
X:154527122	rs61042368		0.03	G/A
X:153554404	rs766420	Intron variant	0.452	C/G
X:153626649	rs915941	′U TR variant	0.275	A/C
X:153626738	rs915942	Splice region variant	0.245	G/A
X:153827637	rs4898389	Intergenic variant	0.312	G/A
X:153828269	rs5986877	Intergenic variant	0.319	G/C
X:153834100	rs7053878	Upstream gene variant	0.191	A/T
ND	b36_153426256			C/T
X:153760429	b36_153413623		0.026	G/A
X:75555627	rs35228794		0.332	C/T
X:153760953	rs2230036	Synonymous variant	0.034	C/T
X:154529974	rs2515906		0.205	A/G
X:154531643	rs1050757	3′UTR variant	0.327	T/C
X:153761628	rs5986990	Non coding exon variant	0.11	G/A
X:153753490	rs28470352	Intergenic variant	0.113	T/A
X:153829693	rs7879049	Upstream gene variant	0.49	G/A

a- Chromosome position, b-Reference SNP identifier (ID number), c-Minor Allele Frequency, d-Reference (Major Allele) and Alternate (Minor Allele), ND (not determined)

Table 10:-G6PD Mutations Identified from all study sites, Ethiopia, 2017

Study Site M:F (N)	CareStart™ G6PD Phenotype (N)		*Sequencing of the two targets (N=222)*					
			G6PD G202A Frequency			*G6PD A376G* Frequency		
	Normal	Deficient	F(N)		M (N)	F(N)		M(N)
	M:F	M:F	Hom.	Het.	Hemi.	Hom.	Het.	Hemi.
Gambella; 27:22	20:20	7:2		1		1	3	4
Benishangule; 25:17	23:17	2:0					6	1
Gomma; 20:19	20:19	-					5	
Bato Degaga; 27:10	27:10	-			4	1		4
Jawi; 11:7	11:7	-				1		
Ahuri; 9:10		-					2	
Andassa; 9:9	9:9	-				2	1	

Foot note to table 10: M (Male), F (Female), M: F (No of samples in Male to Female ratio), (N) (refers Number), Het. (Heterozygote), Homo. (Homozygote), Hemi. (Hemizygote)

The results of Pearson's chi-square and logistic regression association tests between polymorphic markers and asymptomatic malaria infection, summarized in Table 11, showed that ten markers significantly associated.

Table 11: Association of the polymorphic SNPs and the malaria infection status as detected by nPCR, Ethiopia, 2017

	Pearson's Chi-Square		**Logistic regression**	
SNP	Chi^2	P-value	COR (95% CI)	P-value
rs1050828	0.5370	0.464		
rs1050829	0.5370	0.464		
rs1894260	0.2096	0.647		
rs73573478	5.2198	**0.022**	0.313 (0.111- 0 .884)	**0.028**
rs762515	0.4400	0.507		
rs111827785	4.4611	**0.035**	0.445 (0.207- 0.9546)	**0.038**
rs2515904	0.1930	0.660		
rs2515905	0.2050	0.651		
rs762516	0.0542	0.816		
rs2071429	1.1867	0.276		
rs113492957	5.6734	**0.017**	0.30 (0.106- 0 .846)	**0.023**
rs2004651	8.2525	**0.004**	4.404 (1.489- 13.02)	**0.007**
rs2230037	0.0192	0.890		
rs60030796	1.5949	0.207		
rs766420	4.5402	**0.033**	0.430 (0.195- 0.947)	**0.036**
rs915941	2.2818	0.131		
rs915942	2.9479	0.086		
rs4898389	5.1740	**0.023**	2.502 (1.115 - 5.613)	**0.026**
rs5986877	4.2605	**0.039**	2.202 (1.028 -4.714)	**0.042**
rs7053878	0.7360	0.391		
rs35228794	3.6940	0.055		
rs2230036	1.7831	0.182		
rs2515906	7.0540	**0.008**	4.007 (1.349- 11.897)	**0.012**
rs1050757	5.6133	**0.018**	2.548 (1.156- 5.617)	**0.020**
rs5986990	0.8233	0.364		
rs28470352	0.4934	0.482		
rs7879049	2.1968	0.138		
rs2071429	1.1867	0.276		
b36_153426256	4.1329	**0.042**	0.339 (0.115- 1.001)	**0.050**
b36_153413623	0.1126	0.737		

Table 12: Polymorphic SNPs associated with asymptomatic malaria infection as detected by nPCR

SNPs	Allele (Alt/Ref)	Individuals carrying major allele and malaria infected %(n/N)	Individuals carrying minor allele and malaria infected %(n/N)
rs73573478	G/A	83 (32/39)	17 (7/39)
rs111827785	T/C	63 (24/38)	37 (14/38)
rs113492957	C/T	82.5 (33/40)	17.5 (7/40)
rs2004651	T/G	89.5 (34/38)	10.5 (4/38)
rs766420	C/G	27 (10/37)	73 (27/37)
rs4898389	G/A	76.3 (29/38)	23.7 (9/38)
rs5986877	G/C	72 (28)	28 (11/38)
rs2515906	A/G	90 (35/39)	10 (4/39)
rs1050757	T/C	74 (28/38)	26 (10/38)
b36_153426256	C/T	84 (32/38)	16 (6/38)

5.6.1 Identification of markers in linkage and haplotype in the population

Single Nucleotide Polymorphisms with minor allele frequencies greater than or equal to1% were selected to generate Linkage Disequilibrium (LD) across the gene using the software Haploview. High LD was observed between several pairs of SNPs (Fig.11).

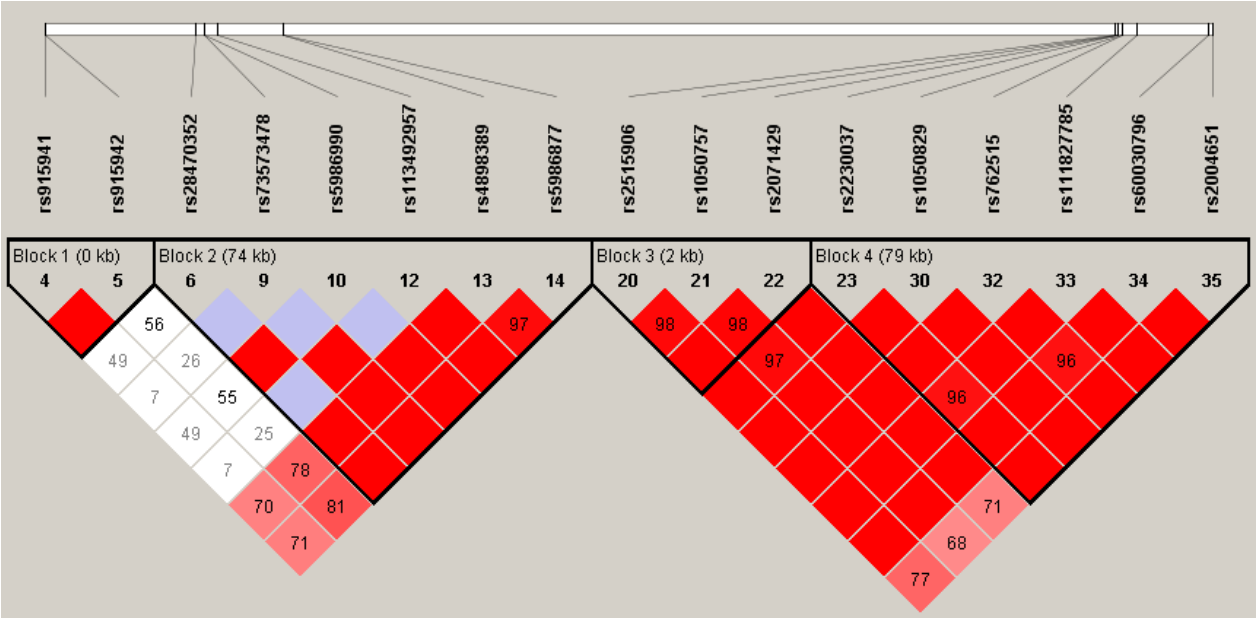

Figure 11: Linkage disequilibrium plot for the whole population

Legend to figure: Numbers in each box represent x D'value, Red square (D' = 1 and LOD = 2), Blue color (D' = and LOD < 2), white color (D' < 1 and LOD < 2), shades of pink or red color (D' < a nd D =)

All tested SNPs were used to identify haplotypes in the population. The algorism described by Gabriel et al. (Barrett et al., 2004) was used to define Haplotype blocks. Haplotypes were estimated using an accelerated Expectation Maximization (EM) algorithm described by Qin et al. in 2002.

Four haplotype blocks were identified. The first haplotype block consisted of two SNPs that are rs915941 and rs915942. The second haplotype block consisted of 6 SNPs that are rs28470352, rs73573478, rs5986990, rs113492957, rs4898389 and rs5986877. The third haplotype block consisted of rs2515906, rs1050757 and rs2071429. Other six SNPs rs2230037, rs1050829, rs762515, rs111827785, rs60030796 and rs2004651 were identified in block four. The recombination rate between blocks was 0.57 for block 1 and 2, 0.94 for block 2 and 3 and 0.87 for block 3 and 4.

Table 13: Haplotype frequency (HF) per each SNP blocks the G6PD gene, Ethiopia, 2017.

Blocks	SNPs						HF
Block 1							
	rs915941	rs915942					
Haplotypes	A	G					0.724
	C	A					0.248
	C	G					0.029
Block 2							
	rs28470352	rs73573478	rs5986990	rs113492957	rs4898389	rs5986877	
Haplotypes	T	G	G	C	G	G	0.496
	T	G	G	C	A	C	0.311
	A	G	A	C	G	G	0.114
	T	A	G	T	G	G	0.062
	T	G	G	C	G	C	0.012
Block 3							
	rs2515906	rs1050757	rs2071429				
Haplotypes	A	T	G				0.664
	G	C	A				0.206
	A	C	A				0.125
Block 4							
	rs2230037	rs1050829	rs762515	rs111827785	rs60030796	rs2004651	
Haplotypes	A	A	T	T	A	T	0.397
	G	A	T	T	A	G	0.223
	G	A	T	T	A	T	0.194
	G	A	T	C	A	T	0.075
	G	G	C	C	G	T	0.069
	G	G	C	C	A	T	0.035

6. Discussion

Ethiopia has enjoyed the tremendous success in control against malaria. Though, it remains to be one of the top ten public health threats and malaria risk is still associated with socioeconomic conditions, failures in bringing adequate behavioral changes in use of preventive measures and lack of sterile cure. Knowledge gaps should be identified not only by researchers, but also by national malaria control programs, and other stakeholders to develop effective interventions strategies for controlling. And having options of sterile cure would greatly accelerate the progress towards the eventual elimination of malaria.

The areas selected for this G6PDd screening study included areas that have high, moderate and low Annual parasite incidence rate with *P.vivax*, *P.falciparum* and mixed infection with the aim of looking into the prevalence and a possible association of G6PDd with malaria incidence.

Asymptomatic parasite carriage estimated by RDT and PCR was very high and as expected; there was significant difference among study sites. This difference in the prevalence might be attributed to the environmental factors, climate and altitude.

From sites in Gambella regional state; the overall *P. falciparum* infection detected was 22.00% and 30.38% by RDT and PCR respectively. Mixed *P. vivax* and *P. falciparum* infection was detected in 5.50% and 3.8% by RDT and PCR respectively; yet no *P.vivax* mono infection was detected. This result supports a previous report among malaria suspects in the same area (Tsegaye *et al*., 2014) which reported 96.9% (257/266) malaria cases were *P.falciparum*. From sites in Benishangule Gumuz regional state the overall *P.falciparum* infection detected was 8.48% and 18.5% by RDT and PCR respectively and mixed infection was below 2% both by RDT and PCR. As compared to sites in Gambella, relatively high (7.41%) *P.vivax* infection was detected with PCR. This result revealed that *P.falciparum* was the predominant *Plasmodium* species detected which is consistent with the study in Yaso district, Kemashi zone (Wale and Mindaye, 2016).

The highest (14.29%) *P.vivax* infection was detected in Bato Degaga village of Adama district, Oromiya regional state. This finding strengthens previous study in East Shewa zone in malaria endemic areas located in the Great Rift Valley (Tadesse *et al*., 2017a) that reported 111 (54%) *P.vivax* cases among 204 microscopically confirmed malaria parasites. In Gomma (Oromiya) malaria infection was very low which is not comparable to 6.3% (112/604) overall prevalence of malaria (Degefa *et al*., 2015) in four villages of Jimma town.

From the rest sites in Jawi, Ahuri and Andassa malaria infection was almost non which is less comparable to the report by (Tadesse *et al*., 2017b) in the same area. On the other hand this result supports the sharp decline of both the numbers of malaria cases and the positivity rates (Toyama *et al*., 2016) in Burie Zuria (West Gojjam Zone), Mecha (West Gojjam Zone), Dembia (North Gondar).

This is the first study performed to evaluate the prevalence of G6PDd in a large number of individuals through phenotypic and genotypic analysis in malaria endemic regions from different agroecological zones of Ethiopia. In this approach qualitative analysis was used in order to gain insights into the frequency of allelic variants associated with G6PDd in those study areas.

The overall phenotypic prevalence of G6PDd was 1.37%. Thirteen (1.50%) males and 9(1.21%) females were deficient for the CareStart test and there was no significant difference between two sexes. This is relatively lower compared to the finding in Tanzania (Mwaiswelo *et al*., 2016) which reported 15.2 % (33/217) G6PDd among *P. falciparum* infected patients and higher than 0.6 % (15/142) G6PDd in southern Ghana (Amoah *et al*., 2016). The absence of difference in G6PDd between male and female participants was consistent with a study done in Sri Lanka (Gunawardena *et al*., 2017). The current study demonstrated that the prevalence of G6PDd significantly varied among different study sites in Ethiopia which is in agreement with (Ghimire et al., 2017) findings. The highest of all 4.00% prevalence was found in Gambella. This result is consistent with those of the previous study by (Tsegaye *et al*., 2014b) reported 7.3% absence of enzyme activity in the same region.

The current study findings showed that G6PDd is more prevalent in Agnuwak ethnic groups compared to others by CareStart G6PDd diagnostic method. G6PDd prevalence was 6.5% (13/200) in Agnuwak ethnic groups, 1.5% (3/200) in Neur ethnic groups, 0.84% (2/239) in Berta ethnic groups and 0.87% (4/484) in Oromo ethnic groups. The overall G6PDd phenotype prevalence was statistically associated with ethnicity (P=0.001). This result is in line with study in Colombia (Valencia *et al*., 2016) that reported different enzymatic activity among different ethnic groups.

Another important finding of this study was identification of variants within the G6PD gene present in a population living in malaria endemic different epidemiologic areas in Ethiopia. In the Ethiopian context, so far no information exists on G6PD gene polymorphism and its association with malaria. Out of total of 36 single nucleotide polymorphisms analysed, common variants like Mediterranean type (rs5030868), Mexico City (rs137852328), rs5030869, rs76723693,

rs137852318, and rs12389569 were not found to be polymorphic in the studied Ethiopian population. A similar finding has been reported previously on the Mediterranean variant from different part of Ethiopia (Tadesse *et al*., 2015b).

Six markers, rs2230037, rs113492957, rs2004651, rs61042368, rs5986990 and rs7879049 were found to have MAF greater than 10%. However, the alternate allele was the minor allele in all those SNPs and four markers were in high linkage disequilibrium (LD). Among markers with minor allele frequency (MAF) below 10%, rs915941 and rs915942, were in complete linkage disequilibrium in block one. Similar finding were reported in Mali (Maiga *et al*., 2014) and in Sri Lanka (Dewasurendra *et al*., 2015). The possible explanation for those two markers being in complete LD could be their close proximity in their location on the chromosome.

Linkage disequilibrium plot was only for the whole population. It is not stratified based on gender, ethnicity and malaria infection status. This is due to small samples genotyped and which would not be more informative to do such classification in this sample size.

It is noteworthy that, two G6PD gene variants have been found in this study. 376AG mutation was relatively higher than 202GA. Total of 31/222 (14%) individuals had mutation for $376A^{+}$ variant. This result is lower than 24% G6PDd reported in 245 individuals in Uganda (Bwayo *et al*., 2014). However, it is higher than 3.6% in 664 individuals in Venezuela (Vizzi *et al*., 2016a) and 1.8% in 1,437 individuals in Gambia (Okebe *et al*., 2014b). The allelic G6PDd frequency was different across study sites. The proportion of mutation in hemizygous males, heterozygous females and homozygous females was 29%, 54.84% and 16.13% respectively. The allelic G6PDd frequency was different across study sites. It was high in Gambella (25.8%) and Benishangule Gumuz (22.58%). Similar study was reported in Thai and Burmese populations in malaria endemic areas of Thailand (Phompradit *et al*., 2011).

It can be noticed that G6PDd is not exclusively in males. This study revealed that out of 31 individuals that had A^{+} mutation, 71% (5 homozygous and 17 heterozygous) were females. In line with this finding high G6PDd allelic frequency in females than males were found in Honduras (Zúñiga *et al*., 2015). Interestingly, 5 individuals (four hemizygous males and 1 homozygous female) found for 202 GA mutations also had 376 A^{+} mutation.

Out of eleven phenotypically deficient individuals, only one hemizygous male was found to have $376A^{+}$ gene mutation (11 were unsuccessfully sequenced). There was discordant between phenotypic G6PDd and genotyping characterization. One plausible reason for those phenotypically deficient individuals being genetically normal and vice versa could be lies on the

sensitivity of the Carestart™ G6PD test that it may have low sensitivity in detecting mild and intermediate G6PD deficiency. The report in Brazil on Validation of the rapid test Carestart™ G6PD (Brito *et al*., 2016) is in support with this premise. Moreover, all G6PD gene variants were not sequenced and those phenotypically deficient individuals might be of those variants not sequenced in this study.

Differences in G6PDd phenotypes and genetic polymorphisms with asymptomatic malaria have been observed. While there was no statistical association between G6PDd phenotype and asymptomatic malaria infection (P>0.05), ten markers were found to be associated with asymptomatic malaria. Out of those, five SNPs, rs2004651, rs4898389, rs5986877, rs2515906 and rs1050757 seem to have a protective effect. And, individuals caring b36_153426256, rs73573478, rs111827785, rs113492957 and rs766420 seem to have higher risk acquiring asymptomatic malaria infection. Some particular factors for this protective variation throughout multiple SNPs could be the age of mutation, type of mutation, and the protective effect of each SNP. The protective effect may also differ on the severity of malaria infection. G6PDd is believed to confer protection against *Plasmodium falciparum* malaria (Clarke *et al*., 2017) and sever malaria (Manjurano *et al*., 2015), however the precise nature of the protective effect has proved difficult to define as G6PD deficiency has multiple allelic variants with different effects in males and females.

The present study found high Genetic diversity across the G6PD gene in the Ethiopian study population. Among the alleles analysed, the 14% G6PDd of the G6PD A376G variant is similar to that identified in many sub-Saharan African countries (Clarke *et al.*, 2017).

7. Conclusion

Single Nucleotide Polymorphisms within the G6PD gene are described in this study population from Ethiopia. Phenotypic characterizations of G6PDd were done and G6PDd found by the Carestart™ G6PD RDT were relatively less. However, Common G6PD gene variants found in Sub-Saharan African countries were also detected with greater frequency.

The findings of this study carry public health policy implication. The establishment of primaquine use for radical cure of *P.vivax* and effective treatment for all *Plasmodium* species should be up on the base line information on the genotypic characteristics of the population. Large scale genetic mapping of the population is needed beyond phenotype characterization so as to avoid the dose dependent risk of haemolysis by using 8-aminoquinoline drugs in G6PDd individuals.

8. Limitation of the study

It is a known that anaemia is common confounder of G6PD screening. However, no adjustment was done for this study and other hematological parameters like hemoglobin level was not measured. Even though, the Carestart™ G6PD test has considerably easier to handle in the field compared to other G6PDd screening tests, it has few limitations. The first is that due to the facts that it does not show a normal control-window and that color development persists for only 10 minutes. Another important limitation is that the test window may display different shades of purple, introducing subjectivity into interpretation of the test results. This may lead to mislabeling those with intermediate and low enzyme activity. For the purpose of this study the interpretations of the test result was done independently by technicians and they were trained in the qualitative test analysis. Though, the high discordance between phenotype and genotype results might be due to the above mentioned reasons.

9. Recommendation

Single Nucleotide Polymorphisms considered in this study provide a starting point for large-scale genetic mapping and presence of these common African gene variants implicates the need for further studies in broad range of ethnic groups to investigate G6PDd. Further investigations on detailed genetic analyses with functional enzyme studies considering both sexes for all asymptomatic, uncomplicated and severe malaria cases should be available in the Ethiopian population.

Indeed, before the administration of primaquine it is necessary to implement a point-of-care screening test for G6PDd detection to avoid complications related to primaquine antimalarial therapy in malaria-endemic regions of Ethiopia. Detailed documentation on G6PD status that reveal more information is required to guarantee the safety of these individuals.

10. Reference

AL-MUSAWI, B. M., AL-ALLAWI, N., ABDUL-MAJEED, B. A., EISSA, A. A., JUBRAEL, J. M. & HAMAMY, H. 2012. Molecular characterization of glucose-6-phosphate dehydrogenase deficient variants in Baghdad city-Iraq. *BMC blood disorders,* 12**,** 1.

AMOAH, L. E., OPONG, A., AYANFUL-TORGBY, R., ABANKWA, J. & ACQUAH, F. K. 2016. Prevalence of G6PD deficiency and *Plasmodium falciparum* parasites in asymptomatic school children living in southern Ghana. *Malaria journal,* 15**,** 388.

ASHLEY, E. A., RECHT, J. & WHITE, N. J. 2014a. Primaquine: the risks and the benefits. *Malar J,* 13**,** 1186.

ASHLEY, E. A., RECHT, J. & WHITE, N. J. 2014b. Primaquine: the risks and the benefits. *Malaria journal,* 13**,** 418.

BAIDJOE, A., STONE, W., PLOEMEN, I., SHAGARI, S., GRIGNARD, L., OSOTI, V., MAKORI, E., STEVENSON, J., KARIUKI, S. & SUTHERLAND, C. 2013. Combined DNA extraction and antibody elution from filter papers for the assessment of malaria transmission intensity in epidemiological studies. *Malaria journal,* 12**,** 272.

BAIRD, J. K. 2015a. Point-of-care G6PD diagnostics for *Plasmodium vivax* malaria is a clinical and public health urgency. *BMC medicine,* 13**,** 296.

BAIRD, J. K., DEWI, M., SUBEKTI, D., ELYAZAR, I. & SATYAGRAHA, A. W. 2015. Noninferiority of glucose-6-phosphate dehydrogenase deficiency diagnosis by a point-of-care rapid test vs the laboratory fluorescent spot test demonstrated by copper inhibition in normal human red blood cells. *Translational Research,* 165**,** 677-688.

BAIRD, K. 2015b. Origins and implications of neglect of G6PD deficiency and primaquine toxicity in *Plasmodium vivax* malaria. *Pathogens and global health,* 109**,** 93-106.

BANCONE, G., CHU, C. S., SOMSAKCHAICHAROEN, R., CHOWWIWAT, N., PARKER, D. M., CHARUNWATTHANA, P., WHITE, N. J. & NOSTEN, F. H. 2014. Characterization of G6PD genotypes and phenotypes on the northwestern Thailand-Myanmar border. *PloS one,* 9**,** e116063.

BARRETT, J. C., FRY, B., MALLER, J. & DALY, M. J. 2004. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics,* 21**,** 263-265.

BEUTLER, E. 1991. Glucose-6-phosphate dehydrogenase deficiency. *New England Journal of Medicine,* 324**,** 169-174.

BOSMAN, A. & CUNNINGHAM, J. 2013. Proposal for an Evidence Review Group (ERG) on G6PD testing to support increased access to primaquine for radical cure of *Plasmodium vivax* and for malaria chemoprophylaxis. *Geneva: World Health Organization*.

BRITO, M. A. M., PEIXOTO, H. M., ALMEIDA, A. C. G. D., OLIVEIRA, M. R. F. D., ROMERO, G. A. S., MOURA-NETO, J. P., SINGH, N., MONTEIRO, W. M. & LACERDA, M. V. G. D. 2016. Validation of the rapid test Carestart (tm) G6PD among malaria vivax-infected subjects in the Brazilian Amazon. *Revista da Sociedade Brasileira de Medicina Tropical,* 49**,** 446-455.

BROEK, L., HEYLEN, E. & AKKER, M. 2016. Glucose-6-phosphate dehydrogenase deficiency: not exclusively in males. *Clinical Case Reports,* 4**,** 1135-1137.

BWAYO, D., KADDUMUKASA, M., DDUNGU, H. & KIRONDE, F. 2014. Prevalence of glucose-6-phosphate dehydrogenase deficiency and its association with *Plasmodium falciparum* infection among children in Iganga distric in Uganda. *BMC research notes,* 7**,** 372.

CARTER, N., PAMBA, A., DUPARC, S. & WAITUMBI, J. N. 2011a. Frequency of glucose-6-phosphate dehydrogenase deficiency in malaria patients from six African countries enrolled in two randomized anti-malarial clinical trials. *Malaria journal,* 10**,** 241.

CARTER, N., PAMBA, A., DUPARC, S. & WAITUMBI, J. N. 2011b. Frequency of glucose-6-phosphate dehydrogenase deficiency in malaria patients from six African countries enrolled in two randomized anti-malarial clinical trials. *Malar J,* 10**,** 241.

CHEN, I., POIROT, E., NEWMAN, M., KANDULA, D., SHAH, R., HWANG, J., COHEN, J. M., GOSLING, R. & ROONEY, L. 2015a. An assessment of the supply, programmatic use, and regulatory issues of single low-dose primaquine as a *Plasmodium falciparum* gametocytocide for sub-Saharan Africa. *Malaria journal,* 14**,** 1-10.

CLARKE, G. M., ROCKETT, K., KIVINEN, K., HUBBART, C., JEFFREYS, A. E., ROWLANDS, K., JALLOW, M., CONWAY, D. J., BOJANG, K. A. & PINDER, M. 2017. Characterisation of the opposing effects of G6PD deficiency on cerebral malaria and severe malarial anaemia. *eLife,* 6**,** e15085.

DALLOL, A., BANNI, H., GARI, M. A., AL-QAHTANI, M. H., ABUZENADEH, A. M., AL-SAYES, F., CHAUDHARY, A. G., BIDWELL, J. & KAFIENAH, W. 2012. Five novel glucose-6-phosphate dehydrogenase deficiency haplotypes correlating with disease severity. *Journal of translational medicine,* 10**,** 199.

DEGEFA, T., ZEYNUDIN, A., GODESSO, A., MICHAEL, Y. H., EBA, K., ZEMENE, E., EMANA, D., BIRLIE, B., TUSHUNE, K. & YEWHALAW, D. 2015. Malaria incidence and assessment of entomological indices among resettled communities in Ethiopia: a longitudinal study. *Malaria journal,* 14**,** 24.

DEWASURENDRA, R. L., ROCKETT, K. A., FERNANDO, S. D., CARTER, R., KWIATKOWSKI, D. P. & KARUNAWEERA, N. D. 2015. G6PD gene variants and its association with malaria in a Sri Lankan population. *Malaria journal,* 14**,** 93.

DOMBROWSKI, J. G., SOUZA, R. M., CURRY, J., HINTON, L., SILVA, N. R., GRIGNARD, L., GONÇALVES, L. A., GOMES, A. R., EPIPHANIO, S. & DRAKELEY, C. 2017. G6PD deficiency alleles in a malaria-endemic region in the Western Brazilian Amazon. *Malaria Journal,* 16**,** 253.

DOMINGO, G. J., SATYAGRAHA, A. W., ANVIKAR, A., BAIRD, K., BANCONE, G., BANSIL, P., CARTER, N., CHENG, Q., CULPEPPER, J. & EZIEFULA, C. 2013a. G6PD testing in support of treatment and elimination of malaria: recommendations for evaluation of G6PD tests. *Malar J,* 12**,** 391.

DOMINGO, G. J., SATYAGRAHA, A. W., ANVIKAR, A., BAIRD, K., BANCONE, G., BANSIL, P., CARTER, N., CHENG, Q., CULPEPPER, J. & EZIEFULA, C. 2013b. G6PD testing in support of treatment and elimination of malaria: recommendations for evaluation of G6PD tests. *Malaria journal,* 12**,** 391.

FANELLO, C. I., KAREMA, C., AVELLINO, P., BANCONE, G., UWIMANA, A., LEE, S. J., D'ALESSANDRO, U. & MODIANO, D. 2008. High risk of severe anaemia after chlorproguanil-dapsone+ artesunate antimalarial treatment in patients with G6PD (A-) deficiency. *PLoS One,* 3**,** e4031.

FARHUD, D. & YAZDANPANAH, L. 2008. Glucose-6-phosphate dehydrogenase (G6PD) Deficiency. *Iranian journal of public health,* 37**,** 1-18.

FRANK, J. E. 2005. Diagnosis and management of G6PD deficiency. *American family physician,* 72**,** 1277-1282.

GHIMIRE, P., SINGH, N., ORTEGA, L., RIJAL, K. R., ADHIKARI, B., THAKUR, G. D. & MARASINI, B. 2017. Glucose-6-phosphate dehydrogenase deficiency in people living in malaria endemic districts of Nepal. *Malaria journal,* 16**,** 214.

GÓMEZ-MANZO, S., MARCIAL-QUINO, J., ORTEGA-CUELLAR, D., SERRANO-POSADA, H., GONZÁLEZ-VALDEZ, A., VANOYE-CARLO, A., HERNÁNDEZ-

OCHOA, B., SIERRA-PALACIOS, E., CASTILLO-VILLANUEVA, A. & REYES-VIVAS, H. 2017. Functional and Biochemical Analysis of Glucose-6-Phosphate Dehydrogenase (G6PD) Variants: Elucidating the Molecular Basis of G6PD Deficiency. *Catalysts,* 7**,** 135.

GONÇALVES, B. P., TIONO, A. B., OUÉDRAOGO, A., GUELBÉOGO, W. M., BRADLEY, J., NEBIE, I., SIAKA, D., LANKE, K., EZIEFULA, A. C. & DIARRA, A. 2016a. Single low dose primaquine to reduce gametocyte carriage and *Plasmodium falciparum* transmission after artemether-lumefantrine in children with asymptomatic infection: a randomised, double-blind, placebo-controlled trial. *BMC medicine,* 14**,** 1.

GONÇALVES, B. P., TIONO, A. B., OUÉDRAOGO, A., GUELBÉOGO, W. M., BRADLEY, J., NEBIE, I., SIAKA, D., LANKE, K., EZIEFULA, A. C. & DIARRA, A. 2016b. Single low dose primaquine to reduce gametocyte carriage and *Plasmodium falciparum* transmission after artemether-lumefantrine in children with asymptomatic infection: a randomised, double-blind, placebo-controlled trial. *BMC medicine,* 14**,** 40.

GREENE, L. S. 1993. G6PD deficiency as protection against *falciparum* malaria: an epidemiologic critique of population and experimental studies. *American Journal of Physical Anthropology,* 36**,** 153-178.

GROUP, W. W. 1989. Updatel Le point. *Bulletin of U19 World Health Organization,* 67**,** 601-611.

GUNAWARDENA, S., KAPILANANDA, G., SAMARAKOON, D., MADDEVITHANA, S., WIJESUNDERA, S., GOONARATNE, L. V. & KARUNAWEERA, N. D. 2017. Prevalence of G6PD deficiency in selected populations from two previously high malaria endemic areas of Sri Lanka. *PloS one,* 12**,** e0171208.

HOWES, R. E., BATTLE, K. E., SATYAGRAHA, A. W., BAIRD, J. K. & HAY, S. I. 2013a. G6PD deficiency: global distribution, genetic variants and primaquine therapy. *Adv Parasitol,* 81**,** 133-201.

HOWES, R. E., DEWI, M., PIEL, F. B., MONTEIRO, W. M., BATTLE, K. E., MESSINA, J. P., SAKUNTABHAI, A., SATYAGRAHA, A. W., WILLIAMS, T. N. & BAIRD, J. K. 2013b. Spatial distribution of G6PD deficiency variants across malaria-endemic regions. *Malar J,* 12**,** 10.1186.

KONE, A. K., SAGARA, I., THERA, M. A., DICKO, A., GUINDO, A., DIAKITE, S., KURANTSIN-MILLS, J., DJIMDE, A., WALCOURT, A. & DOUMBO, O. 2010.

Plasmodium falciparum clearance with artemisinin-based combination therapy (ACT) in patients with glucose-6-phosphate dehydrogenase deficiency in Mali. *Malaria journal,* 9**,** 332.

KUMAR, P., YADAV, U. & RAI, V. 2016. Prevalence of glucose-6-phosphate dehydrogenase deficiency in India: An updated meta-analysis. *Egyptian Journal of Medical Human Genetics,* 17**,** 295-302.

LUZZATTO, L., NANNELLI, C. & NOTARO, R. 2016. Glucose-6-phosphate dehydrogenase deficiency. *Hematology/oncology clinics of North America,* 30**,** 373-393.

LUZZATTO, L. & SENECA, E. 2014. G6PD deficiency: a classic example of pharmacogenetics with on-going clinical implications. *British journal of haematology,* 164**,** 469-480.

MAIGA, B., DOLO, A., CAMPINO, S., SEPULVEDA, N., CORRAN, P., ROCKETT, K. A., TROYE-BLOMBERG, M., DOUMBO, O. K. & CLARK, T. G. 2014. Glucose-6-phosphate dehydrogenase polymorphisms and susceptibility to mild malaria in Dogon and Fulani, Mali. *Malaria journal,* 13**,** 270.

MANJURANO, A., SEPULVEDA, N., NADJM, B., MTOVE, G., WANGAI, H., MAXWELL, C., OLOMI, R., REYBURN, H., RILEY, E. M. & DRAKELEY, C. J. 2015. African glucose-6-phosphate dehydrogenase alleles associated with protection from severe malaria in heterozygous females in Tanzania. *PLoS genetics,* 11**,** e1004960.

MCDONAGH, E. M., THORN, C. F., BAUTISTA, J. M., YOUNGSTER, I., ALTMAN, R. B. & KLEIN, T. E. 2012. PharmGKB summary: very important pharmacogene information for G6PD. *Pharmacogenetics and genomics,* 22**,** 219.

MINUCCI, A., GIARDINA, B., ZUPPI, C. & CAPOLUONGO, E. 2009. Glucose-6-phosphate dehydrogenase laboratory assay: How, when, and why? *IUBMB life,* 61**,** 27-34.

MONTEIRO, W. M., VAL, F. F., SIQUEIRA, A. M., FRANCA, G. P., SAMPAIO, V. S., MELO, G. C., ALMEIDA, A. C., BRITO, M. A., PEIXOTO, H. M. & FULLER, D. 2014. G6PD deficiency in Latin America: systematic review on prevalence and variants. *Memórias do Instituto Oswaldo Cruz,* 109**,** 553-568.

MWAISWELO, R., NGASALA, B. E., JOVEL, I., GOSLING, R., PREMJI, Z., POIROT, E., MMBANDO, B. P., BJÖRKMAN, A. & MÅRTENSSON, A. 2016. Safety of a single low-dose of primaquine in addition to standard artemether-lumefantrine regimen for treatment of acute uncomplicated *Plasmodium falciparum* malaria in Tanzania. *Malaria journal,* 15**,** 316.

OKEBE, J., AMAMBUA-NGWA, A., PARR, J., NISHIMURA, S., DASWANI, M., TAKEM, E. N., AFFARA, M., CEESAY, S. J., NWAKANMA, D. & ALESSANDRO, U. 2014a. The prevalence of glucose-6-phosphate dehydrogenase deficiency in Gambian school children. *Malar J,* 13**,** 10.1186.

OKEBE, J., AMAMBUA-NGWA, A., PARR, J., NISHIMURA, S., DASWANI, M., TAKEM, . N., AFFARA, M., C SA , S. ., NWAKANMA, D. & D'A SS ANDR , U. 2014b. The prevalence of glucose-6-phosphate dehydrogenase deficiency in Gambian school children. *Malaria journal,* 13**,** 148.

OUATTARA, A. K., BISSEYE, C., BAZIE, B. V. J. T. E., DIARRA, B., COMPAORE, T. R., DJIGMA, F., PIETRA, V., MORET, R. & SIMPORE, J. 2014. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is associated with asymptomatic malaria in a rural community in Burkina Faso. *Asian Pacific journal of tropical biomedicine,* 4**,** 655-658.

PETERS, A. L. & VAN NOORDEN, C. J. 2009. Glucose-6-phosphate dehydrogenase deficiency and malaria: cytochemical detection of heterozygous G6PD deficiency in women. *Journal of Histochemistry & Cytochemistry,* 57**,** 1003-1011.

PHOMPRADIT, P., KUESAP, J., CHAIJAROENKUL, W., RUEANGWEERAYUT, R., HONGKAEW, Y., YAMNUAN, R. & NA-BANGCHANG, K. 2011. Prevalence and distribution of glucose-6-phosphate dehydrogenase (G6PD) variants in Thai and Burmese populations in malaria endemic areas of Thailand. *Malaria journal,* 10**,** 368.

SEGEJA, M., MMBANDO, B. P., KAMUGISHA, M., AKIDA, J., SAVAELI, Z., MINJA, D., MSANGENI, H. & LEMNGE, M. 2008. Prevalence of glucose-6-phosphate dehydrogenase deficiency and haemoglobin S in high and moderate malaria transmission areas of Muheza, north-eastern Tanzania. *Tanzania journal of health research,* 10**,** 9-13.

SHAH, S. S., MACHARIA, A., MAKALE, J., UYOGA, S., KIVINEN, K., CRAIK, R., HUBBART, C., WELLEMS, T. E., ROCKETT, K. A. & KWIATKOWSKI, D. P. 2014. Genetic determinants of glucose-6-phosphate dehydrogenase activity in Kenya. *BMC medical genetics,* 15**,** 93.

SNOUNOU, G., VIRIYAKOSOL, S., JARRA, W., THAITHONG, S. & BROWN, K. N. 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Molecular and biochemical parasitology,* 58**,** 283-292.

STANTON, R. C. 2012. Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. *IUBMB life,* 64**,** 362-369.

TADESSE, F., FOGARTY, A. W. & DERESSA, W. 2017a. Prevalence and associated risk factors of malaria among adults in East Shewa Zone of Oromia Regional State, Ethiopia: a cross-sectional study. *BMC public health,* 18**,** 25.

TADESSE, F. G., HOOGEN, L., LANKE, K., SCHILDKRAUT, J., TETTEH, K., ASEFFA, A., MAMO, H., SAUERWEIN, R., FELGER, I. & DRAKELEY, C. 2017b. The shape of the iceberg: quantification of submicroscopic *Plasmodium falciparum* and *Plasmodium vivax* parasitaemia and gametocytaemia in five low endemic settings in Ethiopia. *Malaria journal,* 16**,** 99.

TADESSE, F. G., PETT, H., BAIDJOE, A., LANKE, K., GRIGNARD, L., SUTHERLAND, C., HALL, T., DRAKELEY, C., BOUSEMA, T. & MAMO, H. 2015a. Submicroscopic carriage of *Plasmodium falciparum* and *Plasmodium vivax* in a low endemic area in Ethiopia where no parasitaemia was detected by microscopy or rapid diagnostic test. *Malaria journal,* 14**,** 1-7.

TADESSE, F. G., PETT, H., BAIDJOE, A., LANKE, K., GRIGNARD, L., SUTHERLAND, C., HALL, T., DRAKELEY, C., BOUSEMA, T. & MAMO, H. 2015b. Submicroscopic carriage of *Plasmodium falciparum* and *Plasmodium vivax* in a low endemic area in Ethiopia where no parasitaemia was detected by microscopy or rapid diagnostic test. *Malaria journal,* 14**,** 303.

TOYAMA, Y., OTA, M., MOLLA, G. & BEYENE, B. B. 2016. Sharp decline of malaria cases in the Burie Zuria, Dembia, and Mecha districts, Amhara Region, Ethiopia, 2012–2014: descriptive analysis of surveillance data. *Malaria journal,* 15**,** 104.

TSEGAYE, A., GOLASSA, L., MAMO, H. & ERKO, B. 2014a. Glucose-6-phosphate dehydrogenase deficiency among malaria suspects attending Gambella hospital, southwest Ethiopia. *Malar J,* 13**,** 438.

UYOGA, S., NDILA, C. M., MACHARIA, A. W., NYUTU, G., SHAH, S., PESHU, N., CLARKE, G. M., KWIATKOWSKI, D. P., ROCKETT, K. A. & WILLIAMS, T. N. 2015. Glucose-6-phosphate dehydrogenase deficiency and the risk of malaria and other diseases in children in Kenya: a case-control and a cohort study. *The Lancet Haematology,* 2**,** e437-e444.

VALENCIA, S. H., OCAMPO, I. D., ARCE-PLATA, M. I., RECHT, J. & ARÉVALO-HERRERA, M. 2016. Glucose-6-phosphate dehydrogenase deficiency prevalence and genetic variants in malaria endemic areas of Colombia. *Malaria journal,* 15**,** 291.

VIZZI, E., BASTIDAS, G., HIDALGO, M., COLMAN, L. & PÉREZ, H. A. 2016a. Prevalence and molecular characterization of G6PD deficiency in two *Plasmodium vivax* endemic areas in Venezuela: predominance of the African A-202A/376G variant. *Malaria journal,* 15**,** 19.

VON SEIDLEIN, L., AUBURN, S., ESPINO, F., SHANKS, D., CHENG, Q., MCCARTHY, J., BAIRD, K., MOYES, C., HOWES, R. & MÉNARD, D. 2013. Review of key knowledge gaps in glucose-6-phosphate dehydrogenase deficiency detection with regard to the safe clinical deployment of 8-aminoquinoline treatment regimens: a workshop report. *Malaria journal,* 12**,** 1.

WALE, M. & MINDAYE, A. 2016. Impact of insecticide-treated bednet use on malaria prevalence in Benishangul-Gumuz regional state, Ethiopia. *Journal of vector borne diseases,* 53**,** 215.

World Health Organization, World Malaria Report 2015. *Search PubMed*, pp.1-157.

World Health Organization, 2015. Guidelines for the treatment of malaria. World Health Organization.

ZÚÑIGA, M. Á., MEJÍA, R. E., SÁNCHEZ, A. L., SOSA-OCHOA, W. H. & FONTECHA, G. A. 2015. Glucose-6-phosphate dehydrogenase deficiency among malaria patients of Honduras: a descriptive study of archival blood samples. *Malaria journal,* 14**,** 308.

Appendix I

SOP for Saponin/Chelex DNA Extraction Method

- **EQUIPMENT & CONSUMABLES**

▪ 2x Scissors (per person)	Bunsen Burner
▪ 70% Ethanol	Small bucket to hold Ethanol (with lid)
▪ Dry heat block/water bath/incubator at 37◦C	Dry heat block or water bath at ◦ C
▪ Racks for 10mL & 50mL tubes	0-200 multichannel pipette
▪ 200-1mL multichannel pipette	200uL and 1mL pipette and pipette tips
▪ 2mL eppendorf tubes	96 well plates 1.2mL + lids
▪ Axygen sealing lids	1L/500mL Bottles
▪ Forceps	Gloves
▪ Thermometer	Vacuum system for aspiration
▪ Bench top centrifuge	

- **REAGENTS**

a) 100% ethanol

b) 0,5% high grade saponin in PBS prepared fresh or stored at 4°C or -20°C (Sigma-Aldrich, Product Number S4521), best to have Saponin solution is at RT before use

c) 1X Phosphate Buffered Saline (PBS) Calcium and Magnesium free pH 7.4, stored at 4°C

d) 6% Chelex-100 (Fisher-Scientific Catalog Number NC 7 6) in DNA'ase/RNA'ase free water stored at room temperature (15mL per plate)

e) Distilled water DNA/RNA'ase free

f) PBS in DNA/RNA'ase free water

- METHODOLOGY

➢ **Cutting**

1. samples were Selected from the -20 fridge & lab bench Cleaned by70% ethanol and lay out M-tork
2. Extraction tubes Labeled and a single blood disc cut using 6mm diameter puncher
3. the puncher and forceps Sterilized by dipping briefly in ethanol, and flaming using the Bunsen burner

- **Extraction**

1. 820µL of a 0.5% Saponin solution were added to every well With 1000uL pipette
2. tubes well tightly closed and Put in a rack on a shaker over night at intermediate speed
3. tubes Removed from shaker and photographed, filter paper elution success (colour) and sample ID visibility were checked
4. tubes Centrifuged for 1 minute at high speed and white filter paper discs Confirmed
5. tubes Opened and 200uL of reddish saponin Transferred to a new 0.5mL deep well plate
6. Aspirate the rest of the saponin from the wells using a vacuum system.
   NB: filter paper discs should not be touched, preferably tips changed after every well, alternatively rinsed in PBS
7. 1 mL of cooled (+4) PBS (no saponin) Added to each tube, shacked for 30 minutes and Incubated at 4°C for 30 minutes
8. To get discs to well bottoms, it was shacked sharply and all PBS Removed using a new tip for each tube
9. using thermometer the water bath Set to 6oC , ensured that the 'overheat temperature' was appropriately high (97oC)
10. 150µL of 6% Chelex solution Transferred into each well using a multichannel pipette
11. Then DNA was Extracted by incubating plates for 4x7 minutes at 95-96oC. After removing each incubation, it was moved to an ice tray and shacked the tubes slightly to move the discs around in the Chelex solution
12. tubes Centrifuged at maximum speed for 5 minutes
13. 80µL of the eluted DNA solution Transferred from the spun tubes to Prepared new plates
14. plates Spin for 10 minutes at high speed and stored at -20 or lower degrees Celsius until further use

Appendix II

MetaPhor® Agarose

Microwave lnstructions for Agarose Preparation

1. A beaker 2-4 times the volume of the solution was selected.
2. chilled 1X or 0.5X electrophoresis buffer and a stir bar Added to the beaker.
3. Slowly sprinkled in the agarose powder while the solution was rapidly stirred.
4. The agarose soaked in the buffer for 15 minutes before heated.
5. The beaker and solution Weighed before heated.
6. Beaker Covered with plastic wrap and Pierced a small hole for ventilation.

Note: For agarose concentrations >4%, the following additional steps were performed to prevent the agarose solution from foaming during melting/dissolution

- ❖ The beaker Heated in the microwave oven on Medium power for 1 minute, removed and the solution allowed sitting on the bench for 15 minutes.

7. The beaker Heated in the microwave oven on Medium power for 2 minutes and Removed
8. Gently swirled the beaker to resuspend any settled powder and gel pieces.
9. Reheated the beaker on high power until the solution comes to a boil.
10. Hold at boiling point for 1 minute or until all of the particles were dissolved.
11. Removed the beaker from the microwave oven.
12. Gently swirled the beaker to thoroughly mix the agarose solution.
13. After dissolution, sufficient hot distilled water added to obtain the initial weight.
14. Mixed thoroughly.
15. The solution Cooled at 50°C-60°C prior to casting. Once the gel was casted, the molten agarose cooled and gel at room temperature.

Note: The gel was placed at 4°C for 20 minutes to obtain optimal resolution and gel handling characteristics**.**

Appendix III

Protocol for the preparation of PCR mix for nPCR

Components	Stock concentration	Working concentration	Volume for one reaction (µl)
PCR buffer	5X	1X	5
Separate Mg solution	25mM	µ l	2
dNTPs	25mM	0.25mM each	0.25
Forward Primer	µ M	. µ M	0.625
Reverse Primer	µ M	. µ M	0.625
Taq polymerase	U/ µ l	1U/reaction	0.2
PCR grade water	-	-	11.3
Master Mix Final Volume			µ l
Template	ng / µl	ng /µl	µ l
Total reaction volume			µ l

Appendix IV

Master Mix preparation protocol for PCR -RFLP

Component	Stock Concentration (µl)	Working concentration (µl)
PCR Buffer (X)	5	1
MgCL2 (mM)	25	2
dNTPs (µM)	25	0.25
Primer forward (µM)	10	0.33
Primer reverse (µM)	10	0.33
Taq DNA Polymerase (unit/ µl)	unit / µl	1
Master Mix Total volume		25
DNA Sample (µl)		5
Total reaction volume (µl)		30

Annex I: Information Sheet English version

Title of the study

The prevalence of Glucose-6-Phosphate Dehydrogenase Deficiency among Apparently Healthy Individuals in Selected Malaria Endemic Areas from Different Agroecological Zones of Ethiopia using Phenotyping and Genotyping approaches

Purpose of the study

Ethiopia has enjoyed a remarkable decrease in malaria incidence and mortality in the last decade. However, the glucose-6-phosphate dehydrogenase (G6PD) deficiency which is the most common disease-producing enzymopathy in humans, affecting 400 million people worldwide is one challenge in malaria elimination efforts done. The high prevalence of this inherited disorder among people at risk of malaria, along with the practical difficulty of its diagnosis where almost all of them live, sharply limits the otherwise enormous public health importance and utility of primaquine in controlling and eliminating malaria. Therefore the aim of this study is to investigate G6PD deficiency using both phenotyping and genotyping approaches to safely administration of the drug in malaria prevalence areas of Ethiopia.

Indeed this study provides highly relevant information for national malaria control and elimination efforts; findings are likely to be of value for further researches conducted. We therefore ask your permission to donate your finger prick blood sample. These samples will only be used for this research purpose. In case of further research on stored samples, all identifying information such as your name or address will be removed from the data and ethics approval will be sought.

Annex II:

Discomforts and Risks

You might feel a small amount of discomfort during blood sampling and you may have a small amount of bruising or bleeding where the blood sample was taken. This is considered not to be harmful. We will use sterile equipment to collect the blood sample and the small wound that may arise from the procedure will be treated adequately. The volume of blood is too small to influence your health and the blood will quickly be replaced by your body.

Confidentiality

The records concerning your participation are to be used only for the purpose of this research project. Your name will not be used on labels on laboratory specimens or in any report resulting from this study. At the beginning of the study, we will give you a study identification number and this number will be used on the forms and on the laboratory specimens. Any information obtained in connection with this study will be kept strictly confidential and under lock and key.

Freedom to ask questions

If you have any question concerning this study, do not hesitate to contact the investigator of the study, Getasew Shitaye Ayalew, School of Medicine, Department of medical biochemistry, and Addis Ababa University.

Cell phone: +251913272345

Email:getashitaye@gmail.com

Results from the study will be communicated to your community.

Annex III. Consent Form

I have read the information sheet concerning this study (or have understood the verbal explanation) and I understand what will be required of me and what will happen to me if I take part in it. I also understand that any time I may withdraw from this study without giving a reason and without me or my families' are being affected for my refusal.

May I continue the interview?

1. Yes _____Continue the interview

2. No _____Stop the interview and thank the respondent

Witness's signature certifying that the informed consent has been given

Witness's signature _____ Date _____

Introduction to the Interview

Thank you for deciding to participate in this study. Previously (on the statement of consent form), we have discussed briefly on the purpose of the research, how you were identified, and your part in the research study

Annex IV: Questionnaire Date: __ __/__ __/__ __ __ __ (dd/mm/yyy)

Background Information

. P articipant's code __ __/__ __ __ __/ __ __ __

. P articipant's name _____

3. Age (in years) _____

4. Gender: a. Male b. Female

5. Religion _____

6. Ethnicity _____

7. Region _____

8. Woreda (district name_____

9. Name of town or village_____

10. Kebelle _____

11. Village _____

12. Households number _____

13. Does your house have any of the following?

a) Electricity:

i. e s ii. No iii. Don't know

b) Radio:

i. e s ii. No iii. Don't know

c) Television:

I. Yes ii. No iii. Don't know

14. What type of roof was used for the construction of the house?

a) Grass thatch c) wood and mud

b) Iron sheet d) other, _____

15. What type of wall was used for the construction of the house?

a) Wooden plastered with clay or mud d) Brick or stone

b) Mud with cement plastering e) other, _____

c) Iron sheet

6. W hat is the main material of the house's floor?

a) Earth d) Wood

b) Local dung plaster e) other, _____ c) Cement

17. Are the eaves open or closed?

a) Open c) Partially open

b) closed d) No eaves/not applicable

18. Do you know the causes and transmission of malaria?

a) yes b) no

19. Do you know prevention methods of malaria?

a) yes b) no

20. Have you (has the child) had malaria in the last three weeks?

a) e s b) No c) don't know

21. If yes, when exactly? _____

mpartial witness's name _____ signature_____

Date_____

Researcher's name _____ signature _____Date_____

የመረጃ ማሰባሰቢያ ቅጽ የአማርኛ ትርጉም

የጥናቱ ርዕስ፡-በኢትዮጵያ የወባ ስርጭት በሚገኝባቸዉ የተለያዩ መልክዓ ምድራዊ ቦታዎች በሚኖሩ ጤነኛ የአካባቢዉ ተዎላጆች ላይ የ G-6-PD ኢንዛይም እጥረት መኖሩን ለማወቅ የሚደረግ ጥናት ።

የጥናቱ ዓላማ

ባለፉት አስር ዓመታት ተጨባጭ የሆነ የወባ ስርጭትና በወባ ምክንያት የሚከሰት ሞት መቀነስ ታይቷል። ይሁንና ከፍተኛ የወባ ስርጭት ባለባቸዉ የተለያዩ የዓለማችን ክፍሎች የሚገኘዉና ከ400 ሚሊዮን ህዝብ በላይ የሚያጠቃዉ የ G-6-PD ኢንዛይም እጥረት ወባን ጨርሶ ለማጥፋት በሚደረገዉ ጥረት ላይ ተፅዕኖ በመፍጠር ላይ ይገኛል፡፡ በፆታ ወሳኝ ዘረመል አማካኝነት የሚተላለፈዉ ይህ የ ኢንዛይም እጥረት ምልክቶችን ለማጥናት ካለዉ ዉጣዉረድ በተጨማሪ ፕሪማኪዊን የተባለዉን የወባ ተህዋስያንን ጨርሶ ለማጥፋት የሚያገለግለዉን መድሃኒት እጥረቱ ባለባቸዉ ሰዎች ላይ መጠቀም የተለያዩ የጤና እክሎችን በመፍጠር ህይዎትን እስከማሳጣት ይደርሳል፡፡ ስለዚህ የዚህ ጥናት ዓላማ በ ጤነኛ ሰዎች ላይ የ G-6-PD ኢንዛይም እጥረትን የተለያዩ ዘዴዎችን በመጠቀም መመርመር እና የተጠቀሱትን የወባ መድሃኒቶችን ባግባቡ መጠቀም የሚያስችል መረጃ መፍጠር ነዉ፣ በተጨማሪም የዚህ ጥናት ዉጤት ወባን ለመቆጣጠርና ለማጥፋት ለሚደረገዉ ጥረት እንዲሁም ለሌሎች ቀጣይ ጥናትና ምርምሮች ተገቢ የሆነ መረጃ ይሰጣል ::

ስለሆነም ለዚህ ጥናት ዓላማ ብቻ የሚዉል ከ አንድ ጣትዎ ለአንድዚዜ ብቻ የሚወሰድ አነስተኛ የደም ጠብታ ይለግሱን ዘንድ ፈቃድዎን እንጠይቃለን፡፡

የምቾት መጓደልና ተጋላጭለት

ከልጅዎት የደም ናሙና በሚወሰድበት ገዜ የተወሰነ የምቾት መጓደል ሊኖር ይችላል፤ ደም በተወሰደበትም ቦታ የተወሰነ የመቅላት ወይም መድማት ሊኖር የችላል። ይህ ክስተት ግን ጎጂ እንዳልሆነ ይታሰባል። ናሙናዎችን ለመውሰድ ከጀርም የጸዱ መገልገያዎችን እንጠቀማለን፤ ከናሙና መውሰድ ጋር በተገናኘ ሊከሰት የሚችልን ቁስለት በተገቢው እናክማለን። የሚወሰደው የደም መጠን በጤና ላይ ተጽኖ ለማስከትል በጣም ትንሽ ነው፤ በሰውነት በቶሎ ይተካል።

ሚስጥርን ስለመጠበቅ

ከልጅዎና ከርሶ መሳትፍ የሚገኙት መረጃዎች ለዚህ ጥናት ጠቀሜታ ብቻ ይውላሉ። የርሶም ሆነ የልጅዎት ስም ለናሙና መለያነት ወይም በማንኛውም የዚህ ጥናት ውጤት ሪፖርት ላይ አንጠቀምም። በጥናቱ መጀመሪያ ለተሳታፊዎች የመለያ የሚስጥር ቁጥር ይሰጣል፤ ይህም ለናሙናዎችና በጥናቱ በጥቅም ላይ ለሚውሉ ቅጾች መለያነት ይጠቅማል። ማንኛውም ከዚህ ጥናት በተዛመደ የሚገኝ መረጃ በሚስጥር ይያዛል፤ መረጃዎችም በቁልፍ ተቆልፎባቸው ይቀመጣሉ። የርስዎንም ሆነ የልጅዎን ስም ከጥናቱ የሚስጥር ቁጥር ጋር የሚያገናኘውን መረጃ የጥናቱ ዋና ተመራማሪዎች ብቻ ናቸው ማየት የሚችሉት።

የጥናቱ ተሳታፊ የስምምነት መግለጫ ቅጽ

በኢትዮጵያ የወባ ስርጭት በሚገኝባቸዉ የተለያዩ መልከዓ ምድራዊ ቦታዎች በሚኖሩ ጤነኛ የአካባቢዉ ተዎላጆች ላይ የ G-6-PD ኢንዛይም እጥረት መኖሩን ለማወቅ የሚደረግ ጥናት፡፡

እኔ _____ (የጥናቱ ተሳታፊ ስም), በአርእስቱ በተጠቀሰው በዋና ተመራማሪው ጌታሰዉ ሽታዬ የሚመራው ጥናት ላይ እንድሳተፍ ተስማምቻለሁ፡- ‘‘በኢትዮጵያ የወባ ስርጭት በሚገኝባቸዉ የተለያዩ መልከዓ ምድራዊ ቦታዎች በሚኖሩ ጤነኛ የአካባቢዉ ተዎላጆች ላይ የ G-6-PD ኢንዛይም እጥረት መኖሩን መርመር ጥናት ።‘‘ ሥለ ጥናቱ ዓላማ፣ ሁኔታ፣ ጊዜ፣ ጥናቱን ለመስራት የሚጠቀሙትን ዘዴዎችና ብልሃቶችን ሊከሰቱ የሚችሉትን አለመመቾቶች ተረድቼ በ _____ገለጻ ከተደረገልኝ በሁዋላ ተስማምቻለሁ፡፡ ስለ ጥናቱ የመጠየቅ እድል ተሰጥቶኛል፤ ለጠየኩዋቸውም ጥያቄዎች ተገቢውን ምላሽ አግኝቻለሁ፡፡ ተጨማሪ ጥያቄዎች ቢኖሩኝ ጌታሰዉ ሽታዬን በ ስልክ ቁጥር +251913272345 ማግኘት እችላለሁ ፡፡ በራሴ ፈቃድና ፍላጎት ካልሆነ በቀር በሌሎች ጫና በጥናቱ መሳተፍ የማልገደድ ና የማይገባ መሆኑን እገነዘባለሁ፡፡

አግኝቻለሁ / አላገኘሁም፤ ስለ ጥናቱ ተገቢውን መግለጫ ማግኘቴን (ያክብቡ)

ተረድቻለሁ / አልተረዳሁም፤ ለአንድ ጊዜ ብቻ ጣቴን በመብጣት ስለሚወሰድ ደም ጠብታ ናሙናና ስለጥናቱ ተጨባጭ ውጤት (ያክብቡ)

ተስማምቻለሁ / አልተስማማሁም፤ የተወሰነ ናሙና ከኢትዮጵያ ውጪ ምርምር እንዲደረግበት (ያክብቡ)

ተስማምቻለሁ / አልተስማማሁም፤ የተወሰነ ናሙና ለተወሰነ ጊዜ እንዲቀመጥና ተጨማሪ ምርምር እንዲደረግበት (ያክብቡ)። ተጨ ማሪ ምርምሮች በተቀመጡት ናሙናዎች ላይ የሚሰራ ከሆነ የስነምግባር ኮሚቴን ይሁንታ የሚጠየቅበት ይሆናል፡፡

ተስማምቻለሁ / አልተስማማሁም፤ መጠይቁን ለመሙላት የቃል ጥያቄ ለማድረግ (ያክብቡ)

ተስማምቻለሁ / አልተስማማሁም፤ በጥናቱ ለመሳተፌ (ያክብቡ)

የተሳታፊ ስም፡- _____ ፊርማ_____ ቀን _____

የገለልተኛ ምስክር ስም፡-_____ፊርማ _____ ቀን _____

የተመራማሪው ሰም፡- _____ፊርማ _____ ቀን _____

የመረጃ ማሰባሰቢያ ቅጽ

የጥናቱ ርዕስ፡-በኢትዮጵያ የወባ ስርጭት በሚገኝባቸዉ የተለያዩ መልከዓ ምድራዊ ቦታዎች በሚኖሩ ጤነኛ የአካባቢዉ ተዎላጆች ላይ የ G-6-PD ኢንዛይም እጥረት መኖሩን ለማወቅ የሚደረግ ጥናት

መረጃ አሰባሳቢው እራሱን እና የጥናቱን ዓላም በማስተዋወቅ እንዲሁም የጥናቱን ተሳታፊዎች ለመሳተፍ ፍቃደኛ መሆናቸውን በማመስገን ይጀምራል። ቀን፡-__ __/__ __/__ __ __ __

1.የተሳታፊው ኮድ፡-_____

2.የተሳታፊው ስም፡-_____

3.የተሳታፊው ዕድሜ (በዓመት) _____

4.ፆታ፡- a.ወንድ b.ሴት

5. አምነት _____

6.ነሳ _____

7. ክልል _____

8. ወረዳ_____

9. የከተማው ወይም የመንደሩ ስም_____

10. መንደር _____

11. የቤተሰብ ቁጥር_____

12. መኖሪያ ቤታችሁ የሚከተሉት አሉት?

a.ኤሌክትሪሲቲ

i. አለው ii. የለውም iii. አላውቅም

b.ሬዲዮ

i. አለው ii. የለውም iii. አላውቅም

c.ቴሌቪዥን

i. አለው ii. የለውም iii. አላውቅም

13.መኖሪያ ቤታችሁ ከምን አይነት ጣራ ነው የተሰራው?

a. ከሳር ክዳን
b. ከቆርቆሮ
c. እንጨት እና ጭቃ
d. ሌላ (ይገለጽ)_____

14.ቤቱ ከምን አይነት ጣራ ነው የተሰራው?

e. ከእንጨት እና ጭቃ ምርጊት
f. ጭቃ በሲሚንቶ ምርጊት
g. ከቆርቆሮ
h. ከድንጋይ ወይም ብሎኬት

i. ሌላ (ይገለጽ)_____

15.የቤቱ ወለል ከምንድነው የተሰራው?

a.ዓፈር c.ሲሚንቶ d.እንጨት (ጣውላ)

b. እበት የተለቀለቀ e.ሌላ (ይገለጽ)_____

16.ጣራው ወደዉጭ ክፍት ነው ወይስ ዝግ?

a. ክፍት b. ዝግ

c.መለስተኛ ክፍት

17.ለራስዎ (ለልጅዎ) አጎበር ይጠቀማሉ?

a. አዎ b.አልጠቀምም c.አላውቅም

18.ስለ ወባ መንስዒና መተላለፊያ መንገዶቹ ያውቃሉ

a. አዉቃለሁ b.አላዉቅም

19.ወባን እንደት መከላከል እንደሚቻል ያውቃሉ

a. አውቃለሁ b. አላውቅም

20. ልጅዎ (እርስዎ) ባለፉት ሶስት ሳምንታት ወባ በሽታ ይዞት ያውቃል?

a. አውቃለሁ b. አላውቅም

መልስዎ አዎን ከሆነ በትክክሉ መቼ ነበረ?_____

21. ህክምና ተሰቶት ነበረ?

a. አዎ, የመድሃኒቱን ስም ይጥቀሱ _____

b. አልተሰጠውም (ኻም)

c. አላውቅም

መጠይቁን የሞላዉ ባለ ሙያ ስም _____ፊርማ _____ቀን _____

መጠይቁ የተሞላበት ማዕከል ስም _____

Afan Oromo version of questionnaire-based interview

Gaafannoo Yaada Guuruf Qopaha'e

Mataduree Qoranichaa:

" tiyoopiyaa keessatti tamsa'inni dhibee busaa iddoo itti argamutti,teessuma lafa garaagaraa keessatti dhalattoota bakka sanaa fi fayya-qabeessa ta'an irratti, hir'inni inzaayimii G-6PD iraaachuu isaa hubachuuf qorannoo godhamu."

Kaayoo Qoranichaa:

Waggoota kurnan darban kanatti biyya keenya kessatti tamsa'innii dhibee busaa fi sababa dhibee busan duuni dhufu hir'isaa dhufufuun isaa qabatamaan mul'ateera. Haata'u malee iddowwan tamsa'inni dhibee busaa olaanaan itti mul'atu addunyaa kana irratti kan argamuu fi namoota miliyoona ol kan miidhu hir'inni inzaayimii G-6PD, dhibee busaa guutummaatti balleessuuf tattaaffii godhamu irratti gufuu ta'aa ira. Sex chromosome kana edhamuun kan daddarbu yommuu ta'u mallattoolee Hir'ina inzaayimii kanaa qorachuuf bu'aa bayii godhamu irratti dabalataan maxxantuu dhibee busaa gonka balleessuuf qoricha prymaquine edhamu namoota hir'ina inzaayimii kanaa qabaniif fayyadamuun, fayyaa irratti dhibee fiduun hanga du'uutti geessisa. Kanaaf kaayyoon qorannoo kanaa namoota fayyaa ta'an irratti hir'ina inzaayimii G-6-PD tooftaalee garaagaraa fayyadamuun qorachuu fi qorichoota dhibee busaa jedhaman kana sirnaan akka itti fayyadamnuuf odeeffannoo uumuu dha. Dabalataan bu'aan qorannoo kanaa tattaaffii dhibee busaa to'achuu fi balleessuu godhamuu akkasumas fuulduraaf qorannoowwan biroo godhamaniif odeeffannoo gaarii kenna. Kanaafuu qorannoo kana qofaaf kan oolu quba keessan tokko irraa al tokko qofa kan fudhatamu dhiiga copha xiqqoo akka laattan eyyama keessan gaafanna.

Hir'inoota fi Miidha'aa isaanii

Mucaa keessan irraa yeroo dhiigni fudatamutti hamma tokko waantotni hin mijatiin tokko tokko mul'achuu ni danda'a. FkF iddoo dhiigni fudhatamee xiqqoo ishee dirmmammaa'uu ykn dhiiguun iraachuu ni danda'a. Kun garuu rakoo akka hinqabanne ni amanama. Dhiiga fudhachuuf meshaalee dhibee kamirraallee walaba ta'an ni fayyadamna. Saampilii fudhacuutiin walqabatee madaan mul'achuu danda'an yoo irate hala gariin ni yaalla. Dhiigni fudhatamu xiqaa waan ta'eef qaama irratti dhiibbaa tokko hin qabu sababni isaas qamni dafee bakka waan buusuufi.

Ichiitii Eeguu

Odeeffanoon isin ykn mucaa keessan irraa argamu faayidaa qorannoo kanatiif qofa ola. Maqaan keessanis ta’e kan mucaa keessanii gabaasa qorannaa kanaa keessatti tasumaa hin fayyadamnu. Jalqaba qoranichaa irratti koodiin hirmaattotaaf ni kennama. Kunis Saampilootaa fi qorannicha keessatti unkoota barbaachisan addan baasuuf ola. Odeeffanooni qorannaa kanaan walitti dhufeenya qaban hunduu icciitin ni qabamu, odeeffanoon kunis saanduqa keessatti uguramanii (qollofamanii) kaa’amu. Maqaa keessanis ta’e kan mucaa keessanii lakkoofsa icciitii qorannichaa fana walqabsiisanii beekuu kan danda’an qorattoota qorannichaa qofa ta’u.

Unka Waliigaltee Hirmaattoota Qorannichaa

tiyoopiyaa keessatti tamsa'inni dhibee busaa iddoo itti argamutti,teessuma lafa garaagaraa keessatti dhalattoota bakka sanaa fi fayya-qabeessa ta'an irratti, hir'inni inzaayimii G-6PD jiraaachuu isaa hubachuuf qorannoo godhamu.

Ani _____ (maqaa maatii/guddistuu mucaa), itti gaafatamummaa guutuu qabuun,ofii koo/mucaan koo mataduree qorannoo "**Itiyoopiyaa keessatti tamsa'inni dhibee busaa iddoo itti argamutti,teessuma lafa garaagaraa keessatti dhalattoota bakka sanaa fi fayya-qabeessa ta'an irratti, hir'inni inzaayimii G-6PD jiraaachuu isaa hubachuuf qorannoo godhamu.**" isa edhu gaggeessa qorannichaa obbo Geetaasaw Shittaayee tiin gaggeefamu akka hirmmatu/ttu waliigaleera. Kaayyoo qorannichaa, haala, yeroo, qorannicha gaggeessuuf toftaale fi mala akkasumas hir'inoota mul'achuu danda'an ibsa _____tiin edda naaf taasifamee booda hubadhee waliigaleera. Wa'ee qorannichaa gaafachuuf carraan naaf kennameera. Gaaffileen gaafadheefis deebiin isaanii argadheera. Gaaffilee dabalataa yoon qabaadhe obbo Geetaasaw Shittaayee tiin lakk. +251913272345 irratti argachuu nin danda'a ykn Hoospitaala Alartii fi "Armauer Hansen Research Institute (AHRI)" tti kan argaman barreessaa koree raawwii kan ta'an sarara bilbilaa 0118- 6 3 tiin argachuu nin danda'a. Yeroo kamiyyuu fedhii hirmaachuu koo kaasuu fi mucaa koo qorannicha irraa deebisuu akkan danda'u nin amana. Garu, fayyina mucaa kootiif jecha ani yaalii dabalataa akka argadhu taasisuuf ogeessa fayyaa gaafachu akkan danda'u nin hubadha.

Waa'ee qorannichaa ibsa argachuu koo : Argadheera/ Hin arganne **(itti maraa):**

Yeroo sadii quba mucaa koo fi irree harka isaa/ishee irraa dhiiga fudhatamu fi waa'ee bu'aa qorannicha: Hubadheera/Hin Hubanne **(itti maraa).**

Itoophiyaatiin ala saampiloota muraasa irratti qorannoon akka gaggeeffamu: Walii galeera/ Walii hin galle **(itti maraa).**

Saampilootni qorannaaf sassaabaman muraasni yeroo muraasaaf akka olka'amuu fi qorannoon dabalataa akka gaggeeffamu: waliigaleera/ walii hin galle **(itti maraa).** Yoo qorannoo dabalataa saampiloota ka'aman irratti kan gaggeefaman ta'e eeyyamni koreen raawwii kan gaafatamu ta'a.

Gaafanoo qorannichaa guutuuf, gafannoo afaanii gochuuf : Walii galeera/ walii hin galle.

Qorannicha irratti mucaan koo akka hirmaatu/ttu: Walii galeera/ walii hin galle.

Maqaa Hirmaataa: _____

Maqaa maatii hirmaataa/ttuu_____

Guyyaa: _____

Odeeffanoon kuni meeshaalee eleektiroonikaa gargaaramuun sassaabama. Namni odeeffanoo kana sassaabus mataa isaa fi kaayyoo qoranichaa ibsuun hirmaattoni qoranichas yaada isaanii fedhiiniin waan kennaniif dursee galateeffachuun eegala.

Guyyaa:-__ __/__ __/__ __ __ __

1. Maqaa Hirmaataa: _____
2. Koodii Hirmaataa : __ __/__ __ __ __/ __ __ __
3. Umurii (waggaan)_____
4. Saala:
   a. Dhiira b. Dhalaa
5. Amantaa:_____
6. Qomoo:_____
7. Naannoo:_____
8. Aanaa:_____
9. Maqaa magaalaaa/baadiyyaa:_____
10. Ganda:_____
11. Baayina maatii:_____

12. Manni keessan kanneen armaan gadii ni qabaataa?

a. Ibsaa eleektiriikaa:

i. Eeyyee ii. Lakki iii. Hin beeku

b. Radiyooo:

i. Eeyyee ii. Lakki iii. Hin beeku

c. Televizninii:

i. Eeyyee ii. Lakki iii. Hinbeku

13. Baaxii manni keessanii maal irraa ijaareame?

   a. Chitaa d. Kanbiroo(haaibsamu)
   b. Qorqoorroo
   c. Mukaa fi dhoqqee

14. Gidgiddaan mana maalirraa hojjetame?

a. Muka dhoqeen maragame
b. Simmintoon garafame
c. Qorqoorroo
d. Xuubii ykn dhagaa
e. Kanbiroo(haaibsamu)

15. Lafti mana keesanii mal irraa tolfame?

a. Biyee
b. Dhoqee looniin lolla'amaa
c. Simintoo
d. Muka
e. Kanbiroo(haa ibsamu)_____

16. Baaxiin ykn xaaraan manaa gara bakkeetti banaa moo cufaadha?

a. Banaadha
b. Cufaadha
c. Walakkan banaadha

17. Matta keessaniif ykn daa'imman keessaniif agoberii ni fayyadamtuu?

a. Eeyyee
b. Lakki
c. Hin beeku

18. Akkaataa Ka'umsaa fi daddarba dhibee busaa ni beektuu?

a. beeka
b. Hin beeku

19. Dhibee busaa akkaataa ittiin ittisuu danda'an beektuu?

a. Beeka
b. Hin beeku

20. Torban sadan darban keessa isin/ijoollee keessan dhibeen busaa qabee beekaa?

a. Eeyyee
b. Lakki
c. Hin beeku

Deebi'iin keessan eeyyee yoo ta'e yoomi?_____

21. Yaaliin isiniif godhameee turee?

a. Eeyyee, maqaa qorichaa ibsaa_____
b. Hin kennamne:_____
c. Hin beeku_____

Maqaa ogeessa Gaafannoo guutee:_____

Mallattoo:_____

Guyyaa:_____

Giddu gala Gaafannoon itti guutame:_____

Declaration

I declare that this research paper titled on **the prevalence of Glucose-6-Phosphate Dehydrogenase Deficiency among Apparently Healthy Individuals in Selected Malaria Endemic Areas from Different Agroecological Zones of Ethiopia using Phenotyping and Genotyping approaches** is my original work and has not been submitted for a degree in any other university, and that all sources of materials used for the research have been properly and suitably acknowledged.

Getasew Shitaye

Signature_____

Date _____