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ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

PREVALENCE OF CHLOROQUINE RESISTANCE ALLELES IN *PLASMODIUM FALCIPARUM* AFTER TWO DECADES OF WITHDRAWAL OF CHLOROQUINE USAGEFOR TREATMENT OF FALCIPARUM MALARIAIN ETHIOPIA

TEMESGEN MENBERU KEBEDE

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ABSTRACT

In response to the emergence of P. falciparum (Pf) drug resistance, Ethiopia had changed anti-malaria treatment guidelines, from chloroquine (CQ) to sulfadoxin-pyrimethamine (SP) combination in 1998as first line drugs for the treatment of Pf malaria. The current status of chloroquine resistance (CQR) alleles in Pf is not known. The available few previous studies focused on clinical isolates. This study was conducted to investigate the prevalence of CQR alleles in Pf in asymptomatic and symptomatic cases. A total of 140 dried blood spot samples (DBS) from microscopy positive clinical patients (n=41) in Adama malaria center and asymptomatic individuals from Gambella (n=72) and Benishangul-Gumuz (n=27) with rapid diagnostic tests (RDT) confirmed Pf mono-infections were collected. The genomic DNA was extracted using saponin-chelex extraction method. It was then screened for the confirmation of Pf with18S nested PCR. Only 80 PCR confirmed Pf mono-infection samples were amplified using nested PCR for the three target genes which are incriminated in determining CQ resistance. These included P. falciparum chloroquine resistance transporter gene codon 76 (Pfcrt 76), P. falciparum multidrug resistance gene-1 codon 86 and 1034 (Pfmdr-1 86 and Pfmdr-1 1034). Single nucleotide polymorphisms (SNPs) of the respective targets were analyzed in Polymerase chain reaction- Restriction fragment length polymorphisms (PCR-RFLP) analysis. The overall prevalence of combined mutations of CQR alleles (Pfcrt K76T and Pfmdr-1 N86Y) was detected in 61.5% (48/78) of successfully amplified and analyzed samples. The Pfcrt K76T (CQR) mutation was detected in 61.3 % (46/75), and the Pfmdr-1 N86Y mutation was found in 2.7 % (2/75). The Pfcrt K76T mutation was found fixed in the majority of 90.9 % (30/33) of the clinical isolates from Adama and its surroundings. A relatively lower Pfcrt K76T allele was found in asymptomatic samples from Gambella and Benishangul-Gumuz with 40.7 % (11/27) and 33.3 % (5/15) respectively. The Pfmdr-1 N86Y mutation was detected in only 7.1% (2/28) of samples from Gambella and has exhibited higher reversal rate in all study sites, with 97.0 % (32/33) in clinical samples from Adama and its surroundings. The reversal rate in the asymptomatic samples was 89.3 % (25/28) from Gambella and 71.4% (10/14) from Benishangul-Gumuz. The Pfmdr-1 S1034C mutation was not detected in all analyzed samples (0/65). The major CQR determinant, Pfcrt K76T and the complementaryPfmdr-1 N86Y mutations showed statistically significant association ($X^2(4) = 19.4$; P=0.001. Although the overall CQR mutation in the analyzed targets showed a higher reversal rate; the major CQR determinant Pfcrt K76T mutation remained fixed among the parasite population in and around Adama. This most probably is because CQ is still provided for the treatment of P. vivax which sympartically occurs with Pf, resulting in co-infections and could be misdiagnosed as P. vivax and hence treated with CQ. As a result, the Pf parasite population will face the CQ pressure favoring the CQR mutations. Therefore, if CQ resistance of Pf has to be reversed for a more effective and affordable

treatment, CQ must be withdrawn from all co-endemic localities. On the other hand, the higher reversal rate of CQR mutations documented in the present study might offer the opportunity to re-introduce CQ in Gambella and Benishangul-Gumuz regions. However; there is a need for continual assessment on the status of CQR alleles in Pf.

***Key words**: Chloroquine, Drug resistance, Chloroquine resistance reversal, P. falciparum chloroquine resistance transporter, P. falciparum multidrug resistance gene-1, P. falciparum, Restriction digestion, Single nucleotide polymorphism.*

DEDICATION

THIS WORK IS DEDICATED TO MY BELOVED WIFE HAWI WOLDEYESUS, WHO HAS BEEN AN INSPIRATION TO ALL MY DEEDS

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TABLE OF CONTENTS

LIST OF FIGURES

Pages

LIST OF TABLES **Pages**

ABBREVIATIONS/ACRONYMS

ABC-	ATP based/dependent cassette transporters
ACTs-	Artemisinin combination therapies
AFLP-	Amplified fragment length polymorphism
AHRI-	Armauer Hanssen Research Institute
AL-	Artemether- lumefantrine
Asn-	Asparagine
Asp-	Aspartic acid
ASRA-	Allele specific restriction analysis
ATP-	Adenosine tri-phosphate
BP-	Base pair
BS-	Blood stage
CRISPR-	Clustered regularly-interspaced short palindromic repeats
Cys-	Cysteine
CQ-	Chloroquine
CQR-	Chloroquine resistant
CQS-	Chloroqiune susceptible
DBS-	Dried blood spot
DMT-	Drug and metabolite transporter
DV-	Digestive vacuole
FDRE-	Federal Democratic Republic of Ethiopia
GMS-	Greater Mekong Sub-region
HEW-	Health extension workers
HRM-	High resolution
DNA	Melting
HRP2-	Histidine rich protein 2
RBC-	Red blood cell
iRBCs-	Infected red blood cells

iRFLP- Inverse restriction fragment length polymorphism

IRS- Insecticide residual sprays

LS- Liver stage

McSNP- Melting curve single nucleotide polymorphism

MNPs- Multi-nucleotide polymorphisms

NGS- Next generation sequencing

nPCR- Nested polymerase chain reaction

PBS- Phosphate buffered saline PCR- polymerase chain reaction

Pi- Isoelectric point

*Pf*crt- *Plasmodium falciparum* chloroquine resistance transporter gene

*Pf*mdr-1- *Plasmodium falciparum* multidrugresistance-1 gene

*Pf*PR- *Plasmodium falciparum* parasite rate

pLDH- Plasmodium lactate de-hydrogenase RDTs- rapid diagnostic tests

REs- Restriction enzymes

RFLP- Restriction fragment length polymorphism RFMT- restriction fragment melting temperature

RPM- Revolution per minute

Ser- Serine

SNPs- Single nucleotide polymorphisms

SNM- Single nucleotide mutation

SP- Sulfadoxine-pyrimethamine

TBE- Tris-Borate electrophoresis buffer

Thr- Threonine

T-RFLP- Terminal restriction length polymorphism

Tyr- Tyrosine

UV- Ultra violet light

1. INTRODUCTION

1.1. BACKGROUND

Malaria remained a disease of public health threat especially in sub-Saharan Africa, despite the success during the past decades which reduced cases and deaths by 48% and 18% respectively (WHO, 2015).According to WHO malaria report; out of 212 million cases of malaria globally, 429,000 lives were lost in the year 2015 of which 70% were children under 5 years. Of the global malaria burden, about 90% of malaria cases and 92% of deaths occurred in sub-Saharan Africa(WHO, 2016; Malaria Fact Sheet, 2016).In the same year Ethiopia reported an estimated 2.8 million cases and 4,900 deaths (WHO, 2016).

Malaria control and elimination efforts are challenged by complex factors that emanate from the vector, the parasite, and the environment. The global agenda to eliminate and ultimately eradicate malaria in a step wise move to create a malaria free world by the end of 2030 and ahead (WHO, 2016) might be ambitious. Anti-malarial drug resistance is one of the challenges posed by the parasite. A lot of work has been done to tackle the disease and contain drug resistant malaria, which shade light on the current insight into the study of the vectors, parasites, diagnostics and drug discovery (Edwards and John, 2016; Burrows *et al.*, 2017). The association of anti-malarial drug resistance and mutations in the *Plasmodium falciparum* (*P. falciparum*) chloroquine resistance transporter gene (*Pf*crt) and multidrug resistance-1 gene (*Pf*mdr-1) (Valderramos and Fidock, 2006) had been well established.

The origin and distribution of the major chloroquine resistance (CQR) haplotypes had been identified and defined, where the wild type CQR haplotype CVIET had been found to be the Southeast Asian and African type. Whereas, the SVMNT CQR haplotype is found mainly in

Asia and South America (Fidock *et al.*, 2000; Awasthi and Das, 2013).Though limited in number there are similar studies conducted in African countries such as Kenya (Muai *et al.*, 2009), Tanzania (Mohammed *et al.*, 2013), Uganda (Ocan and Ogwal, 2016) and Zambia (Mwanza et al., 2016).In Ethiopia, three recent studies conducted on the prevalence of Pfcrt, Pfmdr-1 mutations and major CQR-haplotypes and their distribution by (Mula et al., 2011; Mekonnen et al., 2014; Golassa et al., 2014) showed possible patchy distribution. Similar patchy distribution were reported elsewhere (Pulcini et al., 2015). Golassa et al., (2014) reported 100% fixation of the Pfcrt K76T (CQR/mutant) in samples taken from Western Arsi Zone, Shalla District, Mekonnen et al., (2014) reported about 84.1% of the samples to harbor chloroquine sensitive (CQS/wild type) Pfcrt K76 allele and 85.1% carried the Pfmdr-1 N86 allele (N86/wild type). The report by Golassa et al., (2015) showed that most of the isolates from Ethiopia were mutant for the Pfcrt K76T and wild type for Pfmdr-1 86 alleles (N86).However, the results from the two investigators were not in agreement and their study sites were different.

Furthermore, since most of the previous studies on the prevalence of CQR alleles focused on clinical parasite isolates, studies must include asymptomatic cases with wider geographic coverage to generate information that could give a nationwide picture on the issue.

Studies elsewhere described that asymptomatic cases are serving as a drug resistance reservoirs and source of transmission and expansion (Sturrock *et al.*, 2013; Abdul-Ghani *et al.*, 2017). Asymptomatic malaria has become a serious chronic health problem in endemic community which could be treated if correctly diagnosed. At the same time the extent and burden of asymptomatic malaria has not been fully defined in Ethiopia (Tadesse *et al.*, 2015). Therefore, a study on the magnitude of prevalence of chloroquine resistance alleles in asymptomatic malaria in Ethiopia would serve as a guide to estimating the reservoirs of drug resistant malaria, based on which a drug policy could be adapted to mitigate the problem.

1.2. EPIDEMIOLOGY OF FALCIPARUM MALARIA

The distribution of P. falciparum is typically tropical, limited to areas with a temperature ≥20 0C.The spatial distribution of P. falciparum which was developed in 2010 based on the annual parasite rate (PfPR) depicted that an estimated 2.6 billion people were at risk of infection (Gething et al., 2011). Of the at risk population, more than half (52.0 %) were located in Africa. And the P. falciparum stable transmission area counted for 1.4 billion, of which 753 million African, of the total at risk population. Ethiopia was cited in low stable transmission area with

*Pf*PR$_{2\text{-}10}$ ≤ 5% (Figure 1).

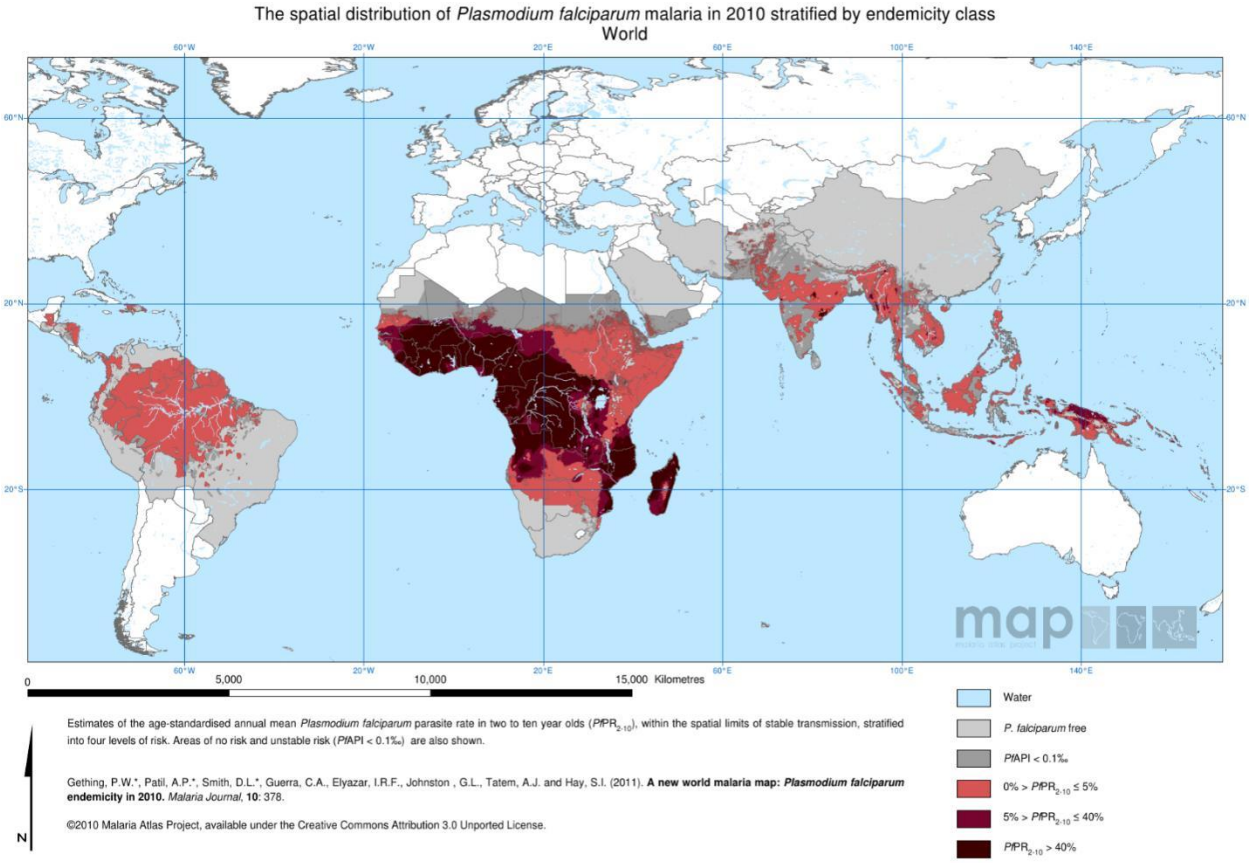


Figure 1: Map showing the geographic distribution of *P. falcparum* malaria (Gething *et al.*, 2011)

1.3. LIFE CYCLE OF PLASMODIUM FALCIPARUM

Plasmodium falciparum (*P. falciparum*) has a digenetic life cycle involving two hosts as shown in Figure 2. The life cycle begins when infected female *Anopheles* mosquito inoculates sporozoites in to the human host during blood meal "#1". Infective sporozoites injected into the skin, start to penetrate and make their ways to the blood vessels within 1-3 hours heading to the liver. The successful sporozoites reaching the liver will infect the hepatocytes and transform to a liver stage (LS) in 2-10 days; start to multiply asexually and increase in number "#2".The LS development ends by the release of up to 40,000 merozoites per infected hepatocyte"#3". The LS merozoites then burst out of the hepatocytes and cross to the blood vessels to infect the RBCs and initiate the blood stage (BS) merozoites. These BS merozoites then undergo a series of asexual reproduction and develop through different stages to form the ring form, throphozoites and schizonts increasing in number to millions and billions. The whole course of intrinsic life cycle "#4" from sporozoite inoculation to the first appearance of BS merozoites will take up to 10 days (Cowman *et al.*, 2016). The schizonts then break burst from the infected red blood cells (iRBCs) every 48 hours to re-infect new RBCs, to continue the second BS cycle.

While in the blood stage, some BS merozoites differentiate and become female and male gametocytes "#5".The matured male and female gametocytes are taken with the blood meal "#6". In the mid gut of the mosquito a diploid zygote is formed by the sexual union of male and female gametes. The zygote differentiates and gives rise to an ookinete. The ookinete burrow through the perithrophic membrane and undergo a series of asexual reproduction to increase in number and form the oocysts. The oocysts develop asexually and penetrate the mid gut wall, attach to the outer mid gut lining and develop into an early sporozoite stage "#7". The sporozoites burst and migrate to the salivary glands through the haemocoel attaining maturity through their journey. Asexual multiplications of the early sporozoites in the salivary glands

increase their number. In 17 days the extrinsic cycle culminates with the production of fully matured infective stage sporozoites. Inoculation of the infective sporozoites up on biting of the female mosquitoes completes the cycle '#8'(Cowman *et al.*, 2016).

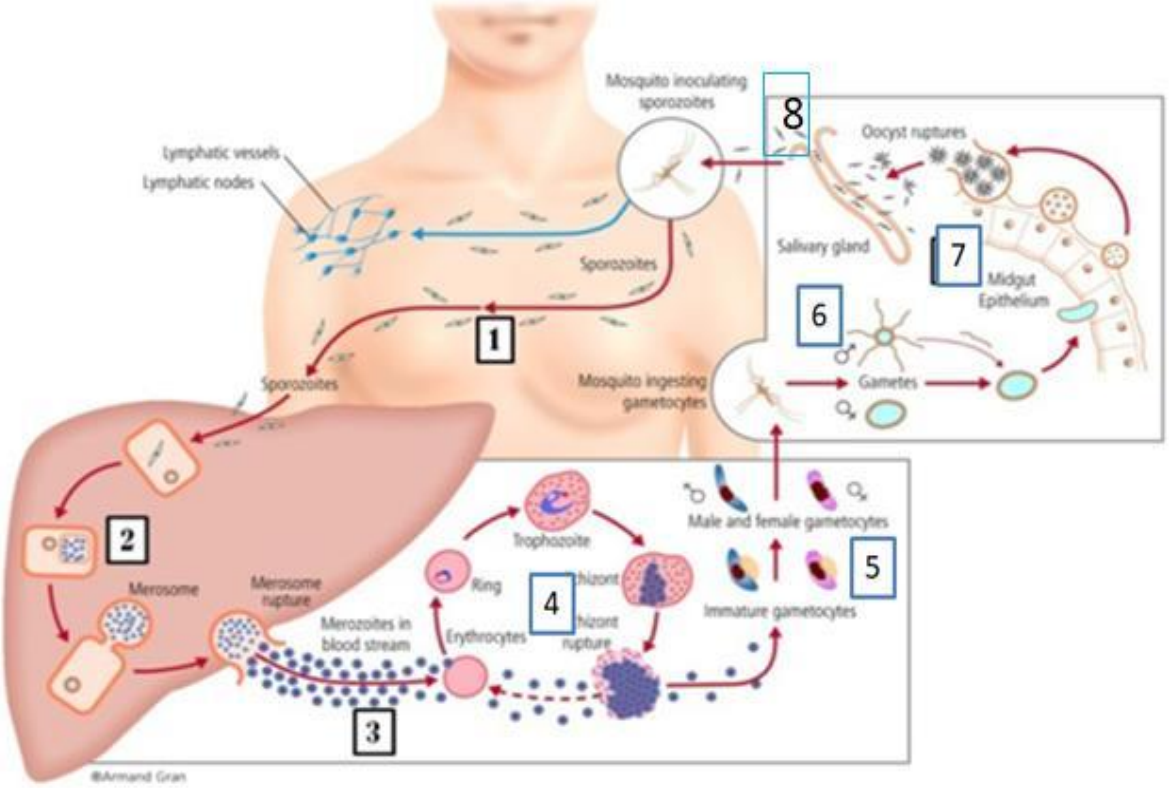


Figure 2: Life cycle of *P. falciparum* (Modified from Google image, Open access sub set of PubMed/PMC)

1.3.1. WHY DRUG RESISTANCE RAPIDLY EMERGE IN P. FALCIPARUM

The frequent and fast development of drug resistance in *P. falciparum* unlike *P. vivax* is mainly due to its transmission strategy. The frequent recombination, alternation between sexual and asexual stage, in *P. falciparum* might increase the frequency of the introduction of mutations and occurrence of higher clonality. In addition, development of male and female gametocytes in *P. falciparum* happen late in the course of the diseases after the peak clinical symptoms has appeared. At this stage, it is almost certain that the patient will be on anti-malarial therapy. Thus, repeated exposure of the gametocytes, the stages responsible to extend the extrinsic life cycle

(mosquito phase), to anti-malarial agents (Price *et al.*, 2014) will increase the probability of selecting few surviving mutant gametocytes that carry resistance alleles. Thus; anti-malarial resistance need to be frequently assessed and effectively detected in *P. falciparum* to tackle the challenges of emergence and spread of drug resistant strains.

1.4. THE PATHOGENESIS OF FALCIPARUM MALARIA

Falciparum malaria is the most severe and life threatening. The severity of falciparum malaria arises from *P. falciparum's* peculiar biology and transmission strategy. *P. falciparum* infection includes inflammation, sequestration and anemia. The inflammatory response is due to the production of pro-inflammatory cytokines in response to the waste and the debris from the periodic rupturing of the red blood cells due to blood stage merozoites. The severe anemia results because of lysis of iRBCs and blockade of blood supply to vital organs due to sequestration and formation of rosette by non-iRBCs around iRBCs in microvasculature (Crompton *et al.*, 2014; Cowman *et al.*, 2016).

1.5. DIAGNOSIS AND TREATMENT

Prompt diagnosis and effective treatment of falciparum malaria are key components of the control, treatment and elimination. Early detection of infection facilitates successful treatment and early cure will shorten the transmission cycle and parasite reservoirs among the community. In the past decades treatment of falciparum malaria has been repeatedly challenged by the rapid emergence of resistance to anti-malarial drugs. In Africa CQR was first reported from the Eastern region, in Kenya (Fogh *et al.*, 1979) and Tanzania (Campbell *et al.*, 1979) and later spread to the Western regions. In response to the development of resistance to CQ by *P. falciparum* many African countries changed their first-line of drug for the treatment of uncomplicated *P. falciparum* malaria from CQ to SP in the early 1990s.

In Ethiopia there was no report of treatment failure of CQ for falciparum malaria until 1985. It was first reported in 1996. A nationwide study conducted in 1996 (Alene and Bennett, 1996) documented high CQ treatment failure rate in an uncomplicated *P. falciparum* malaria prompted treatment policy change. Ultimately; CQ was withdrawn and replaced by SP combination therapy in 1998 as the first-line drug, but retained for the treatment of *P. vivax* malaria until today.

In just about six years, emergence of SP resistant *P. falciparum* was reported (Jima *et al.*, 2005) which lead to a nationwide investigation on the therapeutic efficacy of SP conducted in the late 2003. The study recorded a mean SP treatment failure rate of 35.9% and 71.8% for 14 and 28 days of follow-ups respectively. In 2004 a new treatment guideline was developed and SP was replaced by artemisinin based combination therapies (Artemisinin- Lumefantrine (AL)) as first-line drug for the treatment of *P. falciparum* malaria in Ethiopia (Diagnosis and Treatment Guideline for Health Workers, 2004).

The current drug AL for the treatment of falciparum malaria is also under threat; resistant strains were reported (Dondorp *et al.*, 2009). Also treatment failure has been documented in African countries especially due to the failure of partner drugs like; lumefantrine (Dondorp, *et al.*, 2017). Drug pressure has been identified as one of the major factors for the selection and emergence of resistant parasite strains.

Ethiopia has developed a new malaria control strategy by scaling up and incorporating new developments in the field in a national malaria strategy plan for 2014-2020 (National Malaria Program Monitoring and Evaluation Plan, 2014).Thus, a study on the reservoir status of asymptomatic cases in different epidemiological set ups might add new insights to the control program.

1.6. MOLECULAR BASIS OF DRUG RESISTANCE IN *P.FALCIPARUM*

Drug resistance mainly relies on genetic factors, yet environmental variables and drug pressure that each strain was exposed through time are also important contributors. Drugs should first gain access to their targets in order to exert their action on the pathogen under question. They have different mechanisms of actions and specific targets in the pathogens such as, the digestive vacuole (DV), cytosol, mitochondria, cell membrane and others. The intracellular distribution of any drug depends on the solubility, potential to permeate through the cell membrane and the binding affinity to transporters that regulate drug and molecule trafficking through intracellular compartments.

More than 100 genes (Gardner *et al.*, 2002) in *P. falciparum* genome have been classified as putative transporters. *P. falciparum* transporters are involved in various metabolic pathways and are essential in the uptake of nutrients and the dumping of toxic metabolites. Transporters play a role in reduced anti-malarial susceptibility by reducing concentration in their site of action. In *Plasmodium*, there are two types of transporter mediated xenobiotics trafficking into and out of the DV. These are, the P-glycoprotein related transporters which regulate trafficking of drugs in to the DV compartments that include various adenosine tri-phosphate (ATP) based/dependent cassette transporters; like *Pf* multidrug resistance gene-1 (*Pf*mdr-1), *Pf*-multidrug resistance gene-2 (*Pfmdr*-2) and *Pf*- multidrug resistance associated protein (*Pf*mrp). The second class comprises the drug and metabolite transporter (DMT) system. DMT is represented by *Pf*-chloroquine resistance transporter gene (*Pf*crt) which facilitates the movement of drugs and metabolites towards the extracellular environment (Ibraheem *et al.*, 2014).

The *Pf*mdr-1 is predicted to have 12 trans-membrane domains that localize to the DV membrane with both termini facing the cytosol and identified to be involved in multidrug resistance. In *Plasmodium*, the *Pf*mdr-1 mediates the transport of solutes, drugs and metabolites into the DV

from the cytosol; it also acts as an auxiliary mechanism beside simple diffusion for drug entry into the DV. Five plausible single nucleotide polymorphisms are frequently associated with CQR in *P. falciparum* these are; *Pf*mdr-1 codon 86, 84, 1034, 1042, and 1246 (Fidock *et al.*, 2000).

The *Pf*crt gene is predicted to have 10 trans-membrane domains and localized at the DV membrane with both termini facing the cytoplasm. It performs several functions such as efflux of alkaloids, amine compounds, divalent cations, amino acids and peptides that results from the vacuolar procession of globin. The *Pf*crt gene mainly facilitates the transport of drugs and metabolites away from the DV. It is also suggested to have a role in regulation of H^+ homeostasis. About 32 point mutations have been identified in *Pf*crt gene (Ibraheem *et al.*, 2014).The P^H of the DV is maintained at narrow range about 5.0. The majority of the mutations occur at the cytosolic end of the *Pf*crt region. The substitute amino acids whose isoelectric point (Pi) is more than 5.0; will impart positivity for the channel. When Pi value tends to drop below 5.0, higher channel negativity is conferred, this inconsequence affects CQ exit out of the DV.

1.7. MECHANISMS OF CHLOROQUINE RESISTANCE IN *P. FALCIPARUM*

CQR in *P. falciparum* has been associated with single nucleotide mutation (SNM) in the two transporter genes; the *Pfmdr*-1 and *Pfcrt*. Point mutation in the *Pf*mdr-1 gene an energy driven P-glycoprotein pump which is similar to that is encoded by the mammalian *MDR* gene has been associated with CQR in *P. falciparum* (Djimde *et al.*, 2001). Among the *MDR*-like genes isolated in *P. falciparum,* the *Pf*mdr-1 has been incriminated to confer a competent basis for CQR. Substitution of the amino acids Tyr^{86}, Cys^{1034}, Asp^{1042} or Tyr^{1246} are necessary for CQR (Wellems *et al.*, 1991). Polymorphism in *Pf*mdr-1 gene segregate in to two chloroquine responses where, CQS expressed by 86N while CQR expressed mainly by 86Y.At least four single nucleotide polymorphism (SNP) in *Pf*mdr-1 gene had been largely associated with CQR,

N86Y, N1042D, S1034C and D1246Y where, Asparagine (Asn/N) at codon 86 and 1042, Serine (Ser/S) at codon 1034 and aspartic acid (Asp/D) at codon 1426 of *Pf*mdr-1 protein had been replaced by tyrosine (Tyr/Y), aspartic acid, cysteine (Cys/C) and tyrosine respectively. However, this association may not be always consistent and responsible for all cases of CQR (Wellems *et al.*, 1991). Additional study involving genetic crosses, CQR was found to segregate with Cg2 which is located on chromosome 7 rather than with *Pf*mdr-1 found on chromosome 11.

Single nucleotide polymorphism in *Pf*crt gene isolate in to two different category of drug response, either chloroquine sensitive (CQS), which express the CVMNK haplotype or chloroquine resistance (CQR) which express SVMNT haplotype. The two haplotypes CVIET and SVMNT could be named as CQR-mother haplotypes.

However, there would be other haplotypes often found to segregate among CQR-*Pf*crt populations with low frequencies. The two mother haplotypes have distinct origin and distribution along the endemic regions, where the CVIET haplotype is found in Africa and southeast Asia, while the SVMNT predominantly occur in the south America and Papua New Guinea (Alifrangis *et al.*, 2006).These are mutations placed adjacent to and upstream of the 76^{th}position, at the 72^{nd}, 74^{th} & 75^{th} positions with the mono-morphic 73^{rd}, forms two major CQR- *Pf*crt haplotypes (C^{72}, V^{73}, I^{74}, E^{75}, T^{76}) and (S^{72}, V^{73}, M^{74}, N^{75}, T^{76}) that are frequently found distributed throughout the endemic regions (Ibraheem *et al.*, 2014).

These single nucleotide mutations change the P^{H}, hydrophobicity and isoelectric point which crucially determine the characteristics of the amino acids. The change in amino acid sequence will result to a change in the physiology of the DV (Pulcini *et al.*, 2015).These substitutions will alter the general physicochemical properties of the DV; since the substitute amino acids are more

polar as compared to the formers. The properties of the both substituent and substituted amino acids are summarized in (Table 1).

Table 1: Physicochemical properties of substituent and substituted amino acids involved in mutations of selected target genes

Amino acids	Cods	Acidity	Hydrophobicity index	Isoelectric point
Asparagine	ASn/N	Neutral	-3.5	5.41
Aspartic acid	Asp/D	Acidic	-3.5	2.85
Cysteine	Cys/C	Neutral	2.5	5.05
Lysine	Lys/K	Basic	-3.9	9.6
Serine	Ser/S	neutral	-0.8	5.68
Threonine	Thr/T	Neutral	-0.7	5.6
Tyrosine	Tyr/Y	Neutral	-1.3	5.64

1.8. DETECTION OF DRUG RESISTANCE

There are four basic methods that are routinely used to study anti-malarial drug resistance. These are; (1) *In vivo* (2) *In vitro* (3) Animal models and (4) Molecular characterization; additionally, less rigorous methods have been used such as, case reports, case series or passive surveillance. All methods have their inherent limitations and advantages. The fourth one, molecular characterization is the modern and more precise one over the others in providing accurate information on the subject (Bloland 2001).

Molecular techniques have been modified and updated every time with the advance of knowledge in molecular biology and other related disciplines. Theoretically, the frequency of occurrence of specific gene mutations within a sample of parasites obtained from patients from a given area could provide an indication of the frequency of drug resistance in that area analogous

to information derived from *in vitro* methods. Its advantages include, it needs a small amount of genetic material as opposed to live parasites in other methods, and it is free from host and environmental factors, enables to conduct large number of tests in relatively short period of time. Disadvantages include; need for equipment and training. Different molecular methods are in use to detect drug resistance.

1.8.1. CONVENTIONAL METHODS

PCR-RFLP based analysis is a popular technique for genotyping, identification and differentiation of variability in species. It is suitable to explore single nucleotide polymorphisms (SNPs), multi-nucleotide polymorphisms (MNPs) and micro-indels. The technique is based on the recognition of restriction enzymes (REs) to certain nucleotide sequences will leads to the formation of two or more different sized restriction fragments. PCR-RFLP is relatively inexpensive, do not require sophisticated instruments, and is easy to design experiments and analyze results. The disadvantages are it is inconvenient to run different polymorphism detections in a single experiment, and the lack of specificity of restriction enzymes (REs) when there are several SNPs in a single gene (Rasmussen 2012).

PCR-RFLP involves; PCR amplification of a target sequences, treatment of amplicons with appropriate RE and gel electrophoresis for detection and visualization of restriction fragments. PCR-RFLP also employs various modified techniques for the separation and visualization of restriction fragments like, Polyacrylamide technique, capillary electrophoresis and microchannel electrophoresis which allow high resolving ability than the conventional Agarose gel. Additionally; ultrathin-layer slab gel, capillary and microchip electrophoresis are among the available techniques that can reduce heat distortion of bands, allowed fast separation and high resolution of analytes and offer a high throughput level to PCR-RFLP technique. These techniques especially the microchip format use fabrics which are compatible with 96 and 384

well plates and can be integrated with other steps in the PCR-RFLP procedures (Rasmussen 2012).

In addition; there are several PCR-RFLP techniques where by electrophoretic separation is replaced by gel free methods. These includes; PCR combined with restriction fragment melting temperature (PCR-RFMT) also called melting curve analysis of SNPs (McSNP), amplified fragment length polymorphism (AFLP) which involves amplification of sub set of fragment of genomic DNA using limited sets of generic primers, terminal restriction fragment length polymorphism (T-RFLP) amplification using end-labeled primers followed by restriction enzyme treatment of amplified fragments and electrophoretic separation and finally, invers e PCR based amplified restriction fragment length polymorphism (iRFLP). Though, it involves several steps the PCR-RFLP technique remains extremely valuable technique for genotyping of species specific variations with high sensitivity comparable to the current high throughput method of Pyrosequensing (Zhou *et al.*, 2006).

1.8.2. HIGH THROUGHPUT METHODS

There are a lot of high throughputs methods developed in recent years including modifications of the existing techniques and newly invented techniques for genotyping and analysis of variations among species. Due to the fact that large fraction of a genome is highly repetitive or highly variable especially, the genome of *Plasmodium* species, it has been difficult to precisely analyze variations among species using a short read sequencing, like SNPs and point mutations. Therefore, various sequencing techniques had been developed which enabled whole genome sequencing (WGS), which in practice may not be applied for large number of samples as in surveillances due to high cost. However, it has been possible to sequence targeted sequences (amplicon sequencing) for one or several loci using new generation sequencing (NGS) deep sequencing to assess the genetic diversity in a sample or species (Miles *et al.*, 2016). Sequencing

of DNA polymorphism provides a method for identifying novel drug resistance polymorphisms and validation of molecular genotyping (Miles *et al.*, 2016). Though; the development of discounted bench top sequencers with high turnover rates makes NGS within reach for many malaria endemic countries in the near future; its application is limited in such settings due to cost.

Among the current advances in addition to (NGS), includes real-time PCR based methods (Tajebe et al., 2015), single nucleotide primer extension, high resolution DNA melting (HRM), Taq Man allelic discrimination assay (TAC) (Pholwat et al., 2017), ligase detection reaction fluorescent microsphere (LDR- FM) and the more recent development of the clustered regularly- interspaced short palindromic repeats (CRISPR-cas9) a technique for genome editing and analysis of SNP, MNPs and micro-indels in the genome of a given species (Ng et al., 2016).

1.9. STATEMENT OF THE PROBLEMS

Currently cases and deaths due to malaria had been depressed largely. However; the parasites are in a dynamic condition in the face of the various challenges to guarantee their existence. It is evident that the circulation of the parasite within the asymptomatic community avoids drug exposure and other interventions. These asymptomatic reservoirs are implicated as potential propagation source and challenging the control and elimination program. Also, there is no vaccine and the drugs under the pipeline are limited yet, as a result the development and spread of drug resistance malaria continued to challenge the global effort. Understanding the spatial distribution and dynamics of drug resistance malaria not only helps to understand the population structure but also the possibility of reintroduction of anti-malarials. Thus, this study attempted to investigate the CQR alleles of *Pf* within the asymptomatic and symptomatic malaria patients in different epidemiologic settings in Ethiopia after almost two decades of withdrawal of CQ as first line treatment against falciparum malaria.

Molecular surveillances to monitor drug resistance in *P. falciparum* in Ethiopia are limited. Among the few studies; resistance to artemisinin combination therapies (ACTs) that explored the K13 propeller (Kamau *et al.*, 2015), on CQR alleles by (Mula *et al.*, 2011; Mekonnen *et al.*, 2014; Golassa *et al*, 2014/2015) and on sulfadoxin– pyrimethamine (SP) by (Hailemeskel *et al.*, 2013; Tessema *et al.*, 2015) can be mentioned. Most of these studies focused on symptomatic cases. Yet, studies elsewhere indicated that asymptomatic parasite carriage appeared to be a potential threat to the elimination program and largely became a chronic health problem to people in endemic areas (Tadesse *et al.*, 2015). Learning the extent of drug resistant strains in asymptomatic cases is crucial. In addition, the recent studies from adjacent settings in Ethiopia on CQR-*Pf*crt and CQR-*Pf*mdr-1 alleles in symptomatic cases documented reversal in one area and fixation of CQR alleles in other. Thus, existence of such patchy distributions needs to be verified to align control programs with the specific situation. The data generated in this study provided key information whether there is re-emergence of CQS- *P. falciparum* following the withdrawal of CQ and if CQR alleles have spatial distribution.

1.10. STUDY HYPOTHESIS

H_a: The prevalence of CQR alleles in *P. falciparum* has declined during the past two decades following the withdrawal of CQ from treatment regime.

Statistical representation of Hypotheses: H_0: $P_0=100$; H_a: $P_1 \neq 100$

Where P_0 is the known or estimated proportion from previous studies and P_1 is the estimated proportion to occur in the *P. falciparum* population.

1.11. OBJECTIVE OF THE STUDY

1.11.1. GENERAL OBJECTIVE

- To determine the frequency of chloroquine resistance alleles (*Pf*crt and *Pf*mdr-1) in *P. falciparum* in asymptomatic and symptomatic cases from different epidemiologic settings in Ethiopia.

1.11.2. SPECIFIC OBJECTIVES

- To conduct 18s nested PCR for species confirmation in all samples and nested PCR and consecutive restriction fragment length polymorphism analysis in PCR confirmed *Pf* mono-infection samples.
- To determine single nucleotide polymorphism at *Pf*crt codon 76, *Pf*mdr-1 codon 86 and 1034.
- To determine the frequency of chloroquine resistance alleles among asymptomatic and symptomatic cases.

2. MATERIALS AND METHODS

2.1. STUDY SITES, POPULATION AND DESIGN

Cross sectional studies were conducted in sites with different malaria epidemiologic set ups. Sampling was conducted from August to December; 2016 in selected Woredas (administrative zone equivalent to district) from Gambella Region (Abobo and Lare Woredas), Benshangul- Gumuz Region (Mao-Komo and Menge Woredas), and Adama Woreda (Adama Malaria Center) in Oromia Region.

The study population included passively recruited self-presenting individuals with microscopy confirmed uncomplicated *P. falciparum* malaria exhibiting signs and symptoms of malaria and community recruited asymptomatic individuals with RDT confirmed *P. falciparum* malaria.

Patient group: Recruitment was undertaken at Adama Malaria Center. Adama is located at an altitude of 1,664 meters and 08° 32'N 39° 16'E; 100Km Southeast of Addis Ababa. The annual average precipitation of the Woreda is about 808mm and the annual mean temperature is 20.5^{o}C with 59% average humidity. The malaria center is a facility where free microscopic diagnosis and treatment of malaria are offered. The catchment areas are co-endemic for both *P. falciparum* and *P. vivax*. Patients visit Adama malaria center mainly from the Adama Woreda and neighboring Woredas, and seldom as far as Afar due to the long fame of the center, since the malaria control era of the 1960s.

Asymptomatic group: were involved from Gambella and Benishangul-Gumuz Regions. Woredas were selected for the study after consulting the Regional Health Bureau about their malaria endemicity. Gambella is one of the Regional States of the Federal Democratic Republic ofEthiopia (FDRE), found about 772Km away Southwest of Addis Ababa. Malaria transmission in the region is dominated by *P. falciparum*. Participants of the study were enrolled from the two major ethnic groups (Nuer and Anuak). The Anuak occupy the Anuak zone in Abobo Woreda. Abobo is found about 48Km Southwest of the regional

capital, Gambella town; located at 7 53 51.4242 N and 34 32 20.3712E at Lat. 7.014 and Long. 34.0058. With an average altitude of

474meters above sea level. Four Kebeles were selected for this study (Chebokir, Teign, Mender 17 and Okuna) which are occupied by the indigenous Anuak ethnicity. Lare is one of the Woredas in the Nuer zone. It is found about 82Km West of Gambella town; located at 8 19 51.8118 N and 33 57 24.7854E, at Lat. 8.0114 and Long. 33.0072. It lies at an average altitude of 422meters above sea level. Four Kebeles were selected in the Woreda (Kordeng 01, Kordeng 02, Kurgegn and Kutong).

Benishangul-Gumuz Region is also another Regional State of FDRE; the regional capital is Assosa, which is found 664Km from Addis Ababa to the Western part of the country. Menge and Mao-Komo Woredas were selected for the study after consulting the Regional Health Bureau and previous data. Menge Woreda is found 50Km far from Assosa town, located at10 21 12.241 N and 034 45 47.282E, at Lat. 10.0035 and Long. 34. It lies at average altitude of 1167meterabove sea level. While Mao-Komo Woreda is found some 120Km away from Assosa town, located at 09 22 25.695 N and 034 26 18.296 E, at Lat. 9.007 and Long. 34.005. It lies at average altitude of 1807meter above sea level. Four Kebeles in each Woreda were included in the study; Keshaf, Benieshegol, Kuduyu and Belmeghua Kebeles in Menge Woreda; Taja, Tulu, Yeamesera and Gure Kebelles in Mao-Komo Woreda.

Asymptomatic participants in Gambella and Benishangul-Gumuz were enrolled as part of other ongoing research projects of the study team. The community was called by local leaders, Woreda health bureau officials, health extension workers, personnel from respective Kebele administrations. Individual informed consent was filled-in after explaining study procedures in local languages.

2.2. SAMPLE SIZE DETERMINATION

Sample size was calculated using the statistical method "Two independent samples with dichotomous outcomes" (Sullivan, 2011) given by the formula:

$$n_i = \{p_1(1-p_1) + p_2(1-p_2)\}\left(\frac{Z}{E}\right)^2$$

Where $n_{i=}$ is sample size required in each group (i=1, 2); *P*1and *P*2= are the proportions of success in each comparison group; *Z*= value from the standard normal distribution table, confidence level of 95%; *E*= is the desired margin of error

Assumptions:

- $\alpha = 0.05$
- $\beta = 90$ %.
- Two-sided test

ni= {0.95(0.05)+ 0.95(0.05)} $(1.96/0.05)^2$; (0.0475) + (0.0475)=0.095; (1536.64

X0.095)=1
45.98

Thus: about 146 samples are required to successfully determine the CQR *P. falciparum* in the two comparison groups.

2.3. DATA MANAGEMENT

Socio-demographic, clinical, malariometric data collected during the study period and laboratory generated data were entered in duplicate in the data management unit at AHRI. Data were fed to statistical software, processed and analyzed using STATA 13 (stata Corp., TX USA).

2.4. SAMPLE COLLECTION

Socio-demographic data: A semi-structured pre-tested interview based questionnaire was used to capture detailed socio-demographic, clinical and malariometric data on the days of sample collection from participants and parents/guardians in case of minors. Briefly, a simple set of standardized questions on; recent history of fever, history of taking anti-malarial drugs without diagnosis, proximity to water bodies, bed net availability and use, insecticide residual spraying (IRS), key household socioeconomic variables, materials used for housing construction and household facilities were among the data gathered.

Blood sample collection: A finger prick blood was used to prepare dried blood spots (DBS) on 3MM Whatman filter paper (Whatman, Maidstone, UK).The DBS samples from Adama Malaria

Center were air dried and stored in -20^{o}C freezer in zip-locked plastic bags containing self- indicating silica gel desiccant beads (Geejay Chemicals Ltd). DBS samples from Gambella and Benishangul-Gumuz were air dried, stored in zip-locked plastic bags containing self-indicating silica gel desiccant beads at room temperature. DBS samples were transported at ambient temperature to AHRI and stored at -20^{o}C freezer until being processed to confirm parasite carriage by nested PCR and investigation for SNPs associated with CQR using PCR-RFLP. Detection of malaria parasites carriage with microscopy and RDT:

For the patients group thin and thick blood smears were stained for 10 minutes with 10% Giemsa and thin smear was fixed with methanol for 10 minutes in horizontal position then screened for malaria asexual parasites. Individuals were declared negative when no parasites and gametocytes were detected after reading 100 microscopic fields. Consenting asymptomatic individuals were screened with RDT (First Response Malaria Ag.

PLDH/HRP2 COMBO, Rapid Diagnostic Test from Premier Medical Corporation Ltd.). Those found positive for *P. falciparum* mono- infections were included in the study.

2.5. LABORATORY PROCEDURES

2.5.1. DEOXIRIBONUCLEIC ACID (DNA) EXTRACTION

Total genomic DNA was extracted using saponin-chelex dual extraction procedure as described previously (Baidjoe *et al.*, 2013),with few modifications mentioned in (Tadesse *et al.*, 2015); In brief; 6mM diameter DBS samples on filter paper were punched using stainless steel puncher soaked in 96% ethanol alcohol and burned in a Bunsen burner flame between every cutting to avoid contamination and carryover. The DBS discs were soaked with 820µl of 0.5% saponin solution in 96 deep well plates, mounted and run on a shaker overnight. The plate were removed from the shaker the next day, gently shaken to remove discs stuck to lid, centrifuged for 1 minute at 5,000 revolution per minute (RPM)in a Mega Fuge centrifuge (MEGA FUGE 16R, HERAEUS, Thermo Scientific, Germany). The supernatant (serum) was then transferred in to a new storage plate. The remaining solution was then aspirated using vacuum aspirator and 1 ml of

cooled (+4^{o}C) phosphate buffered saline solution (PBS) was added to each well containing the washed DBS discs, shaken for 30 minutes and incubated at +4^{o}C for 30 minutes then shaken sharply to get the discs to well bottom. All PBS solution were removed and dried with aspirator using new pipette tips to each well.

150µL of 6.0% chelex solution prepared with DNase/RNase free water (Promega Madison, WI USA) was added to each well. 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 at 96^{o}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 in to new labeled storage plates with care not to disturb and take the settled chelex. The eluted DNA was then spun at 5,000RPM for 5 minutes in

a Mega Fuge and stored at -20 ^{o}C until the next

procedure.

Saponin Molecular Biology Grade (SIGMA ALDRICH CHEMIE GMBH) and Chelex-100 (BT chelex 100 Resin Biotechnology grade from Bio-Rad Laboratories, Inc.) were used. The detailed saponin-chelex dual extraction method standard operating procedure (SOP) is attached as annex.

2.5.2. SPECIES CONFIRMATION USING NESTED PCR

P. falciparum parasite carriage was confirmed using nested PCR (nPCR) that targeted the 18S small subunit ribosomal gene (ssrRNA) (Snounou,*et al.* 1993). The first round of amplification of the nPCR protocol targeted a region that is conserved between all *Plasmodium* species. Species specific (Nested 2, N2) reaction was done using the Nested 1 (N1) reaction product as a template using *P. falciparum* specific primer pairs (Table 2).

PCR master mix was prepared in a 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. For N1 PCR, 20µl of the master mix was dispensed into 96 well PCR plates and taken to another room where the DNA templates samples were added to make the final reaction volume 25µl. For the N2 PCR, master mix was prepared in exactly the same way as N1 except *P. falciparum* specific primers were used this time. For 25µl N2 PCR reaction 23µl of the master mix and 2µl of the N1 product were used.

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 and negative controls, alongside nucleic acid free water (SIGMA, Switzerland).

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

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

Target Gene	PCR steps	Primer Sequence	PCR Cycling Condition	mplico n size	References
Genera Plasmodium	N1	5'TTAAAATTGTTGCAGTTA AAACG 5'CTTGTTGTTGCCTTAAAC TTC	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	N2	5'TTAAACTGGTTTGGGAAAACCAAATATATT 5'ACACAATAGACTCAATCATGACTACCCGTC	95^0C-10min [95^0C -60sec, 58^0C -60sec, 72^0C -90sec] 30X, 72^0C -10min	205 BP	
P. vivax	N2	5'CGC T TCTAGCT TAATCCACATAACTGATAC 5'ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	95^0C-10min [95^0C -60sec, 58^0C -60sec, 72^0C -90sec] 30X, 72^0C -10min	120 BP	

Gel electrophoresis:

The PCR products were visualized in 2% agarose (Hi-Res Standard agarose from AGCT Bioproducts Ltd.) gel. 2 gm of agarose was dissolved in 100 ml of 0.5X Tris-Borate (TBE) buffer mixed by shaking then boiled in microwave until it melts completely. After allowing it to cool to hand warm; 0.2 µg/mL final concentration of Ethidium bromide (*Bio-*

Rad Laboratories Inc.) was added; mixed by swirling gently and poured into 26 well comb gel cast and left at room temperature until it fully solidified (for ~ 25 minutes). The gel was removed from the cast and submerged in the electrophoresis apparatus (*BIO-RAD SUB-CELL GT*) which was filled with 0.5XTBE buffer. The comb was removed without damaging the sample wells and 10µl of N2 product was loaded to each well in a specific lay out. Positive controls for *P. falciparum* and *P. vivax*, non-template control and 100bp DNA ladder were loaded (final concentration of ~0.3 to 0.5 µg /well). The samples were then run in an electrophoresis apparatus at 120V for 60 minutes. The gel was removed from the apparatus and put in the camera fitted trans-UV illuminator (*BIO- RAD*) and the gel images was taken and saved in a specific folder in the computer system.

2.6. DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS

The nested PCR-RFLP method was used for detection and analysis of SNPs in the selected target genes. The selected CQR markers *Pf*crt 76, *Pf*mdr1 86 and *Pf*mdr1 1034 were amplified in nested PCR using specific primers for each target as described in (Mekonnen *et al.*, 2014; Oladipo *et al.*, 2015).In brief; the master mix contained; 2mM $MgCl_2$, 0.25 mM of each dNTPs, 0.33µM forward primer, 0.33µM reverse primer and 1UnitTaq DNA polymerase, specific amplification conditions for each target gene are summarized in Table 3.

The nested PCR-RFLP products were first detected in agarose gel electrophoresis to confirm the success of amplification of the desired target size; 2% agarose gel (2 gm agarose powder in 100 mL 0.5X TBE buffer) for the *Pf*crt 76 gene which has a size of N2 product of 145bp, 1.8% (1.8 gm agarose powder in 100 mL 0.5X TBE buffer) for *Pf*mdr-1 86 fragment which has a size of 534bp and 1.5% (1.8 gm agarose powder in 100 mL 0.5X TBE buffer) for the *Pf*mdr-1 1034 fragment which is 864bp in size. Successfully amplified samples were then treated in restriction digestion. The digestion mixture for the three targets

contained; 1 Unit ApoI enzyme (0.1 µl), 1X NEB buffer (2.0 µl), 5µl N2 product (~ 1µg) and the remaining volume were filled with PCR grade water to bring the total reaction volume of 20 µL per reaction for respective SNPs (Table 4).

Table 3: PCR– RFLP: Amplification conditions for N1 and N2 PCR for the *Pf*crt76, *Pf*mdr-1 86 and *Pf*mdr-1 1034 genes and sequence of primer pairs used for subsequent restriction digestion of the target genes.

Target Gene	Primer Sequence	Codons	PCR Cycling Conditi	References
*Pf*CRT 76	N1F:5'-CCGTTAATAATAAATACACG CAG-3'	76	95^0c for 10min (94^0c-30sec;56^0c-	Mekonnen *et al*., 2014; Oladipo *et al*., 2015
	N1R:5'-GGATGTTACAAAACTATAGTTACC-3'			
	N2F:5'-TGTGCTCATGTGTTTAAACTT-3'	76	95^0c for 10min (94^0c-30sec;56^0c-	
	N2R:5'-CAAAACTATAGTTACCAATT TTG-3'			
*Pf*MDR-1 86	N1F:5′-AGGTTGAAAAAGAGTTGAAC-3'	86	95^0c for 10min (94^0c-30sec;55^0c-	
	N1R:5′-ATGACACCACAAACATAAAT-3'			
	N2F:5′-ACAAAAAGAGTACCGCTGAAT-3'	86	95^0c for 10min (94^0c-30sec;55^0c-30sec;60^0	
	N2R:5′-AAACGCAAGTAATACATAAA GTC-3'			
*Pf*MDR-1 1034	N1F: 5′-GTGTATTTGCTGTAAGAGCT-3'	1034	95^0c for 10min (94^0c-30sec;55^0c-	
	N1R:5′-GACATATTAAATAACATGGG TTC-3'			
	N2F:5′-CAGATGATGAAATGTTTAAA GATC-3'	1034	95^0c for 10min (94^0c- 30sec-60^0c-	
	N2R:5′-TAAATAACATGGGTTCTT GACT-3'			

Table 4: Restriction digestion of target genes; digestion conditions for *Pf*crt76, *Pf*mdr-1 86 and *Pf*mdr-1 1034 genes, respective restriction enzymes used, N1 and N2 product and cleaved product size of each targets.

Target Gene	Enzyme	N1 Product	N2 Product	Codon Cleaved	Temperature	Incubation Time	Cleaved Product size	Buffer
*Pf*CRT 76	Apo I #R0566S	206bp	145bp	K76 Wild	50^0C	5 hours	99+46BP	NEB# 3.1 Enzymes and buffer from New England *BioLabs* Inc.
*Pf*MDR-1 86	Afl III #R0541L	578bp	534bp	86Y Mutant	37^0C	12 hours	353+181BP	
*Pf*MDR-1 86	Apo I #R0566S	578bp	534bp	N86 Wild	50^0C	5 hours	250+185+99 BP	
*Pf*MDR-1 1034	Dde I #R0175S	958bp	864bp	1034C Mutant	37^0C	5 hours	191+673 BP	
http://medschool.umaryland.edu/malaria/protocols/; Duah *et al.*, 2013; Das *et al.*, 2014								

Restriction digestions and analysis of SNPs and restriction digestion were performed following a protocol described in allele specific restriction analysis (ASRA) of targeted gene by Maryland University (http://medschool.umaryland.edu/malaria/protocols/) and (Humphray *et al.*, 2007; Duah *et al.*, 2013; Das *et al.*, 2014) with slight modifications. The PCR-RFLP products were analyzed in agarose gel electrophoresis, in exactly the same way as described in previous section, except the concentration of the agarose gel;theN2 PCR-RFLP products of the *Pf*crt 76 (145bp), *Pf*mdr-1 86 (534bp) and *Pf*mdr-1 1034 (864bp)were run with2.0%, 1.8% and 1.5% agarose gel respectively.

Interpretation of nPCR/RFLP (SNPs) analysis was done as follows.

The *Pf*crt K76T mutation was detected using Apo I enzyme (New England *BioLabs* Inc.) which recognize the wild type allele (*Pf*crtK76) and cut into two restriction fragments with a size of 99bp and 46bp. The results were interpreted as; completely cut into two fragments with size of 99bp+46bp as wild type (*Pf*crtK76), those samples with intact 145bp only were taken as mutant (*Pf*crt76T) and those which displayed three fragments with 145bp, 99bp and 46bp were considered as mixed or clonal infection.

The *Pf*mdr-1 N86Y mutation was detected in restriction digestion using Afl III (New England *BioLabs* Inc.) which recognize the mutant (*Pf*mdr-1 86Y) and cut the 534bpN2 product into two fragments with the size of 353bp and 181bp. The results interpreted as; those with tow fragments the size of 353 and 181bp are taken as mutant (*Pf*mdr-1 86Y), those with intact 534bp were counted as wild type (*Pf*mdr-1 N86) and those with three fragments with the size of 534, 353 and 181 were considered as mixed or clonal infection.

Moreover; the wild type (*Pf*mdr-1 N86) target was cross checked using Apo I, which recognizes the wild type *Pf*mdr-1 gene and cut into three restriction fragments with the size 250bp, 185bp, and 99bp up on recognition. Thus; those with three fragments with the size of 250, 185 and 99bp were taken as wild type (*Pf*mdr-1 N86), those only intact N2 product size of 534bp were counted as mutant (*Pf*mdr-1 86Y) and those samples with either two or one digested products of any of the sizes of restriction fragments and the original 534bpwere considered as mixed or clonal infection.

The *Pf*mdr-1 S1034C was digested with the restriction enzyme, Dde I which recognizes the mutant (*Pf*mdr-1 1034C) and cut into two restriction fragments with the size 191bp, and 673bpup on recognition. The results were interpreted as; those with complete digestion and displayed two fragments with191 and 673bp size were taken as mutant (*Pf*mdr-1 1034C), those with intact N2

product size are counted as wild (*Pf*mdr-1 S1034) and those with three bands of two digested fragments with N2 product are taken as mixed or clonal infection.

2.7. ETHICAL CONSIDERATION

The proposal of this study obtained ethical approval from the Institutional Review Board of Addis Ababa University College of Natural and Computational Sciences (CNCS-IRB) on 09/01/2017, Minute number (IRB/024/2017); Ref. number(CNSDO/241/09/2017). The major project under which this project was executed, obtained an ethical approval from the ethics review boards of Addis Ababa University (CNSDO/264/08/16), Jimma University (RPGC/395/06), Armauer Hansen Research Institute (PO52/14), The National Research Ethics Review Committee (310/109/2016) and the London School of Hygiene & Tropical Medicine (10628).

3. RESULTS

3.1. SOCIO-DEMOGRAPHIC, CLINICAL AND MALARIOMETRIC CHARACTERISTICS OF STUDY POPULATION

The characteristics of the study population are summarized in table 5. The median age of the study participants was 16 years (IQR: 10,26).Of the study population 17.0% (7/41) confirmed that they had at least one confirmed malaria episode in the past 12 months, out of which100.0% (7/7) were treated for malaria, of which 57.1% (4/7) were treated with CQ. The total number of responses for the question on taking anti-malarial drugs without diagnosis was 41, of which 7.3% (3/41) reported to have taken anti-malarial drugs without diagnosis. Among the self-presenting symptomatic individuals 70.7% (29/41) had fever, body temperature $\geq 37.5\ ^{0}C$.

Study participants from Adama were also assessed for the availability and utilization of intervention tools. 51.2% (21/41) respondents indicated that they own bed nets out of which66.7% (14/21) replied proper usage. The proportion of bed net to person lies less than 1 bed net per person (65/120=0.5). At the same time only 36.6% (15/41) respondents confirmed the application of IRS in and around their house within the past 12 months.

The majority of participants from Adama 65.0% (26/40) have electricity in their house, with 67.5% (27/40) own radio and 46.3% (19/41) have television. However; 100.0% of respondents from Gambella (69/69) and Benishangul-Guz (27/27) reported to live in a house roofed with grass thatch, wall made of wood plastered with mud and a floor of soil or earth without electricity.

Participants who responded for the presence or absence of water bodies in their vicinity, 34.1% (14/41) confirmed the presence of water body nearby their residence, out of these, 42.9% (6/14) reported the presence of river followed by other type of water bodies.

Table 5: Socio-demographic, clinical and malariometric characteristics of study populations collected during the study from September to December, 2016 from Adama Malaria Center, Gambella and Benishangul-Gumuz Regions.

CHARACTERISTICS	GAMBELLA, % (n/N)	ADAMA, % (n/N)	BENISHANGUL, % (n/N)	TOTAL, % (n/N)
Age in years, median (25th – 75th percentile)	13.0(8,20)	25 (18,35)	12(8,20)	16(10, 26)
Female sex	34.7(25/72)	34.1 (14/41)	37.0(10/27)	35.0 (49/140)
Previous malaria episodes		17.0 (7/41)		
Treatment given		100.0 (7/7)		
Type of drug , CQ		57.1 (4/7)		
Taking anti-malarials without diagnosis		7.3(3/41)		
Intervention utilization				
Ownership of bed nets		51.2 (21/41)		
Bed net/person<1		0.5 (65/120)		
Insecticide residual spraying		55.0 (22/41)		
Fever (temperature ≥37.5 °C)		70.7 (29/41)		
P. falciparum* samples collected by microscopy/RDT				
P. falciparum	72*	41	27*	140
***Plasmodium spp*. Confirmation by nPCR**				
P. falciparum	57.0(41/72)*	68.3(28/41)	59.2(16/27)*	60.7(85/140)
P. vivax	5.6(4/72)	7.3(3/41)	7.4(2/27)	6.4(9/140)
Mixed spp. Infection	4.7(3/72)	12.2(5/41)	0.0(0/27)	5.7(8/140)
Negative samples	33.3(24/72)	12.2(5/41)	33.3(9/27)	27.2(38/140)
Roof type				
Thatch	97.1(68/70)	24.4 (10/41)	77.8(21/27)	71.7 (99/138)
Iron sheet	2.9 (2/70)	75.6 (31/41)	22.2 (6/27)	28.3 (39/138)
Wall Type				
Wooden plastered with mud /clay	100.0 (69/69)	39.0 (16/41)	100.0(27/27)	81.8 (112/137)
Mud with cement		34.1 (14/41)		10.2 (14/137)
Iron sheet		19.5 (8/41)		5.8 (8/137)
Stone or brick		7.3 (3/41)		2.2 (3/137)
Floor Type				
Soil or Earth	100.0 (69/69)	48.8 (20/41)	100.0 (27/27)	84.7 (116/137)
Local dung		44.0 (18/41)		13.1 (18/137)
Cement		4.9 (2/41)		1.5 (2/137)
Others		2.4 (1/41)		0.7 (1/137)
Water Bodies		34.1 (14/41)		
River		42.9 (6/14)		
Pond		14.3 (2/14)		
Stagnant water		35.7 (5/14)		
Swamp		7.1 (1/14)		
Facilities				
Electricity	0.0 (0/70)	65.0 (26/40)	0.0 (0/27)	19.0 (26/137)
Radio	1.4 (1/70)	67.5 (27/40)	14.8 (4/27)	23.4 (32/137)
Television	0.0 (0/69)	46.3 (19/41)	0.0 (0/27)	13.9 (19/137)

3.2. MALARIA PREVALENCE WITH MICROSCOPY AND RDT AND CONFIRMATION WITH NESTED PCR

Among the total of 99RDT confirmed *P.falciparum* mono-infection samples collected from asymptomatic individuals in Gambella and Benishangul-Gumuz, 57.0% (41/72) and 59.2%(16/27)had been confirmed to be *P. falciparum* mono infection up on nested PCR confirmation. Out of microscopy confirmed *P. falciparum* mono-infections samples from Adama, 68.3% (28/41) were confirmed to bear *P. falciparum* mono-infection, up on nested PCR. The gel image of PCR amplification for *P. falciparum* is presented in Figure 3.

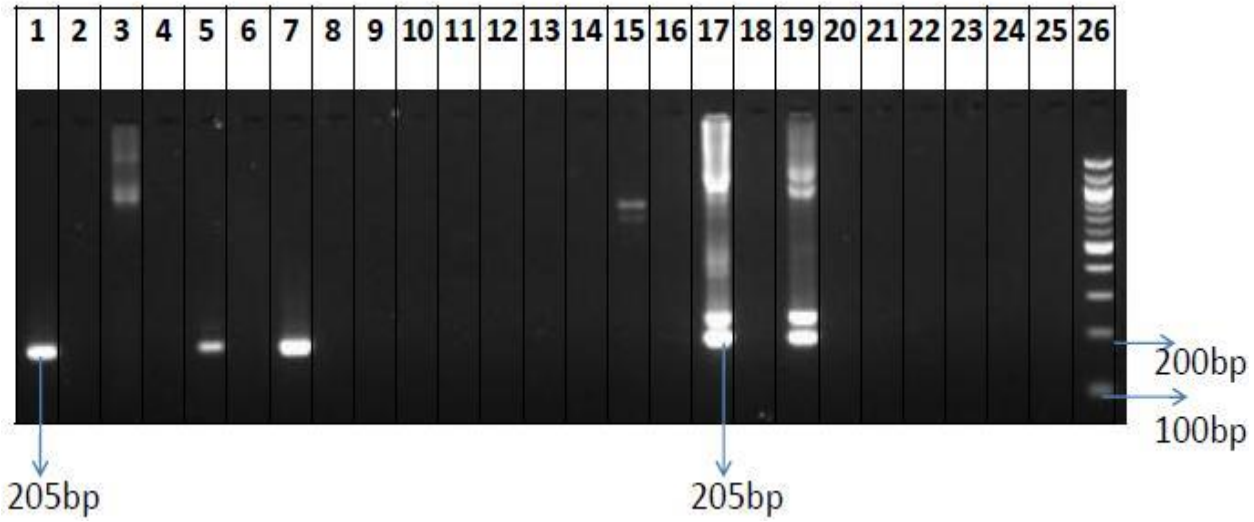


Figure 3: Representative gel image taken at AHRI lab. PCR activity from January to May, 2017 for the nested PCR confirmation of *P. Falciparum* mono infection, the N2 product was run at 120V for 60 minutes using 2% agarose gel prepared with0.5X TBE buffer: lanes1, 3, 5, 7, 9,11,13 and 15 samples, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 25-Empty, 17 and 19-positive controls (NF54) *P. falciparum*, 21and 23non-template control, 26-100bp standard DNA ladder.

3.3. PREVALENCE OF CHLOROQUINE RESISTANCE ALLELES

Samples confirmed to bear *P. falciparum* mono infections up on nested PCR were further analyzed for the four SNPs (*Pf*crt K76T, *Pf*mdr-1 N86Y, *Pf*mdr-1 N86 and *Pf*mdr-1 S1034C) that were previously reported to have strong association with CQR. Of the 80 samples analyzed in PCR-RFLP; 37.5% (30/80), 42.8% (34/80) and 20.0% (16/80) were from Gambella, Adama and Benishangul-Gumuz respectively. Each targeted marker was amplified with a general success rate of amplification of 87.8% (range: 81.3%–93.8%); with 93.8% (75/80), [92.5% (74/80); 82.5% (66/80)] and 81.3% (65/80) for the *Pf*crt 76, *Pf*mdr-1 86 and *Pf*mdr-1 1034 respectively. The expected size of N2 product of each target were effectively amplified and detected; *Pf*crt 76 a size of 145bp, *Pf*mdr-1 86 with 534bp and *Pf*mdr-1 1034 which is 864bp respectively. The representative gel images of PCR amplification products for each target are presented in Figures 4, 5, 6 and 7.

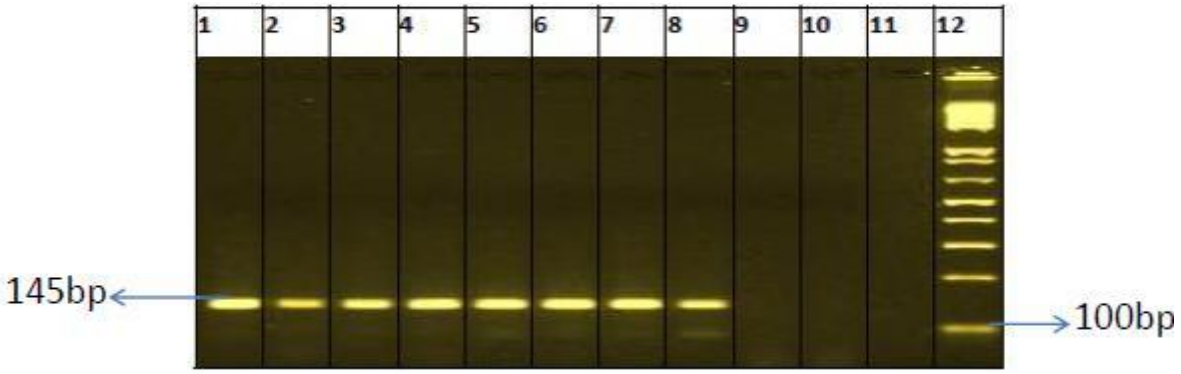


Figure 4: Representative gel images of PCR products for *Pf*crt 76 target taken at AHRI lab. PCR activity from January to May, 2017; Electrophoresis was run at 120V for 60 minutes using 2% agarose gel prepared with 0.5X TBE buffer: *Pf*crt 76 gene (size=145bp); lanes 1-8 = samples, lanes 9 and 10= non-template control, lane 11= empty, lane 12=100bp standard DNA ladder.

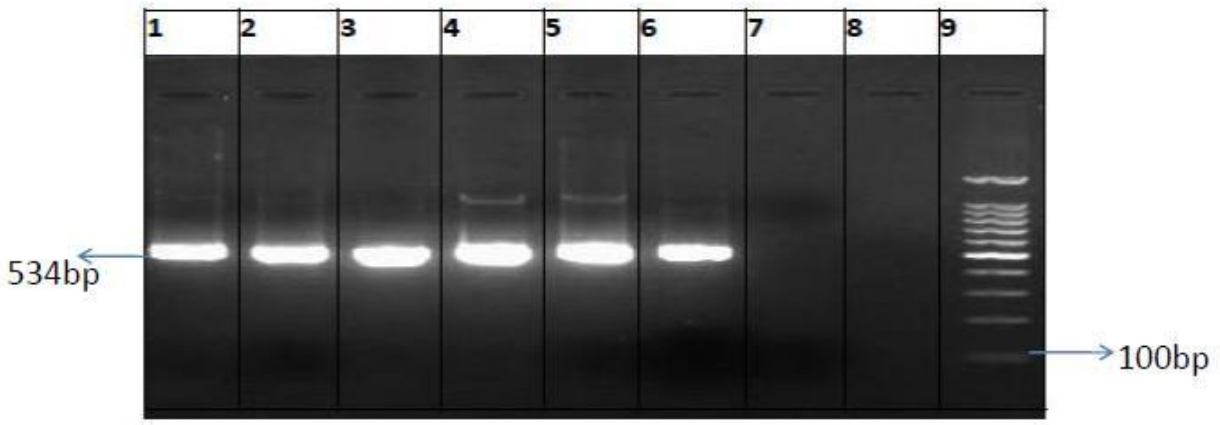


Figure 5: Representative gel images of PCR products for *Pf*mdr-1 86 target taken at AHRI lab. PCR activity from January to May, 2017; Electrophoresis was run at 120V for 60 minutes using 1.8% agarose gel prepared with 0.5X TBE buffer:*Pf*mdr-1 86 gene (size=534bp); 1-6- samples, 7 and 8- non-template control, 9- 100bp standard DNA ladder.

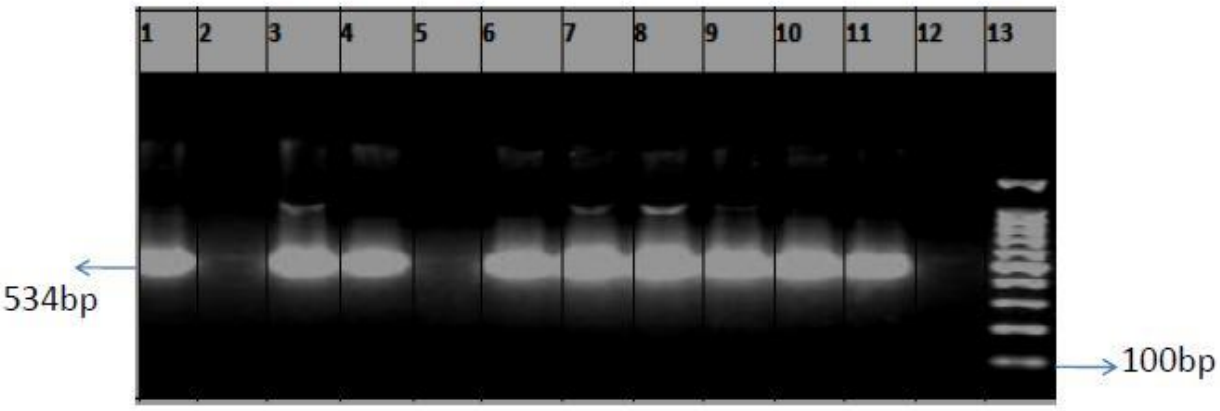


Figure 6: Representative gel images of PCR products for *Pf*mdr-1 86 target taken at AHRI lab. PCR activity from January to May, 2017; Electrophoresis was run at 120V for 60 minutes using 1.8% agarose gel prepared with 0.5X TBE buffer: *Pf*mdr-1 86 (size=534bp); 1- 11-samples, 12- non-template control, 13- 100bp standard DNA ladder.

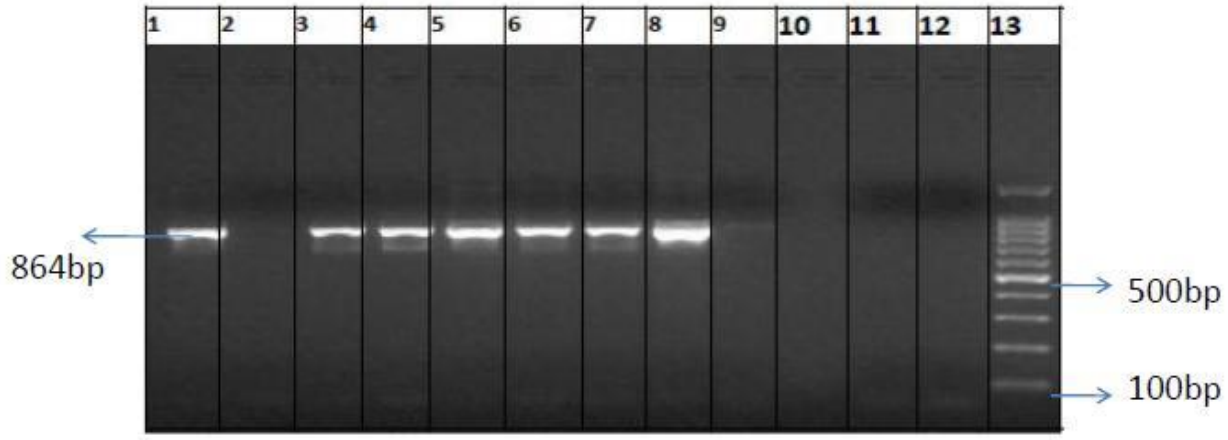


Figure 7: Representative gel images of PCR products for *Pf*mdr-1 1034 target taken at AHRI lab. PCR activity from January to May, 2017; Electrophoresis was run at 120V for 60 minutes using 1.5% agarose gel prepared with 0.5X TBE buffer: *Pf*mdr-1 1034 gene fragment (size=864 bp); 1-9- samples, 10 and 11- non-template control, 12- empty, 13- 100bp standard DNA ladder.

Results of restriction digestion analysis:

Successfully amplified samples with the nested PCR; were processed in restriction digestion with their respective REs for SNP analysis in agarose gel electrophoresis. The *Pf*crt K76T digestion results were interpreted as; those samples with 99bp and 46bp fragments counted as wild type, three bands, one- N2 product (145bp), two- 99bp and 46bp digested products considered as mixed or clonal infection and intact N2 product considered as mutant. In the *Pf*mdr-1 N86Y mutation with digestion of Afl III which recognize the mutant allele were analyzed as; those samples with two bands size of 353BP and 181bp taken as mutant, those with three bands of N2 product (534bp), 353bp and 181bp were counted as mixed infections and intact with only N2 product (534bp) size were taken as wild type. The *Pf*mdr-1 86 target was cross checked with Apo I enzyme which recognize the wild type allele. The results were analyzed as; samples with three fragments of bp 185bp and 99bp counted as wild, those samples with 4 bands of N2 product (534bp) plus fragments with size of 250, 185 and 99 bp considered as mixed/clonal and intact N2 product only are counted as mutants. The *Pf*mdr-1 S1034C mutation was digested using

Dde I enzyme which recognize the mutant allele, but no mutant allele was detected in all analyzed samples (0/65).The prevalence of CQR alleles in analyzed target genes were; 61.3% (46/75) and 2.7% (2/74) for the *Pf*crt K76T and *Pf*mdr-1 N86Y mutations respectively. The *Pf*mdr-1 N86Y mutation was analyzed in restriction digestion using Afl III enzyme which recognize the mutant *Pf*mdr-1 86Y allele. The mutant allele was detected in 2.7% (2/74) of the samples. This mutation was cross-checked with digestion of Apo I enzyme that recognize the wild type (N86) allele. In this analysis the mutant allele was found in 1.5% (1/66). One of the samples which were detected as mutant during the Afl III digestion, was classified to be mixed with two bands (one restriction fragment of size 99bp and intact N2 product of 534bp) during Apo I digestion. The prevalence of the *Pf*mdr-1 N86Y mutation was estimated by combining results from the two analyses, considering samples found to be mutant in both or anyone of the two analyses as mutant. The representative gel images of restriction analysis of each target genes are presented in Figures 8, 9, 10 and 11. Looking at the *Pf*crt K76T and *Pf*mdr-1 N86Y combined mutations we found a significant (Pearson $X^2(4) = 19.4$, P= 0.001) association (Table 6).

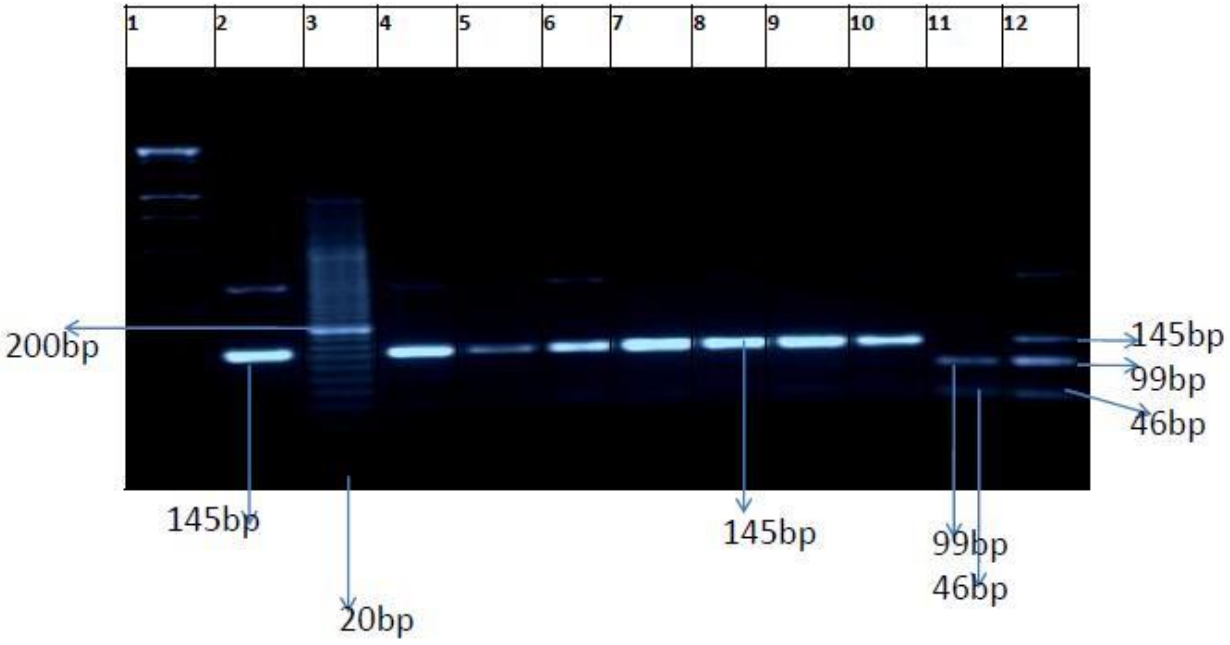


Figure 8: Representative agarose gel images for restriction analysis of the analyzed SNPs taken at AHRI lab. PCR activity from January to May, 2017: *Pf*crt K76T digestion with Apo I enzyme: lane 1= 100BP standard DNA ladder, lane 2= undigested control sample, lanes 3=20BP standard DNA ladder, Lane 4=undigested control sample, lanes 5-12=samples.

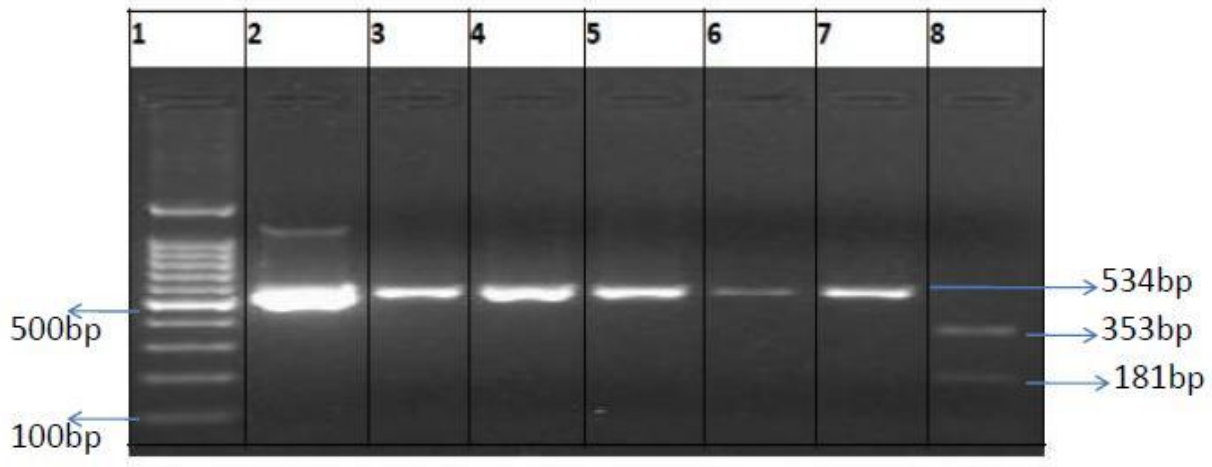


Figure 9: Representative agarose gel images for restriction analysis of the analyzed SNPs taken at AHRI lab. PCR activity from January to May, 2017: *Pf*mdr-1 N86Y digestion with Afl III enzyme: N2 product size=534BP; cleaved product size=353+181BP; lane 1- 100BP standard DNA ladder, 2- undigested control, 3-8- samples.

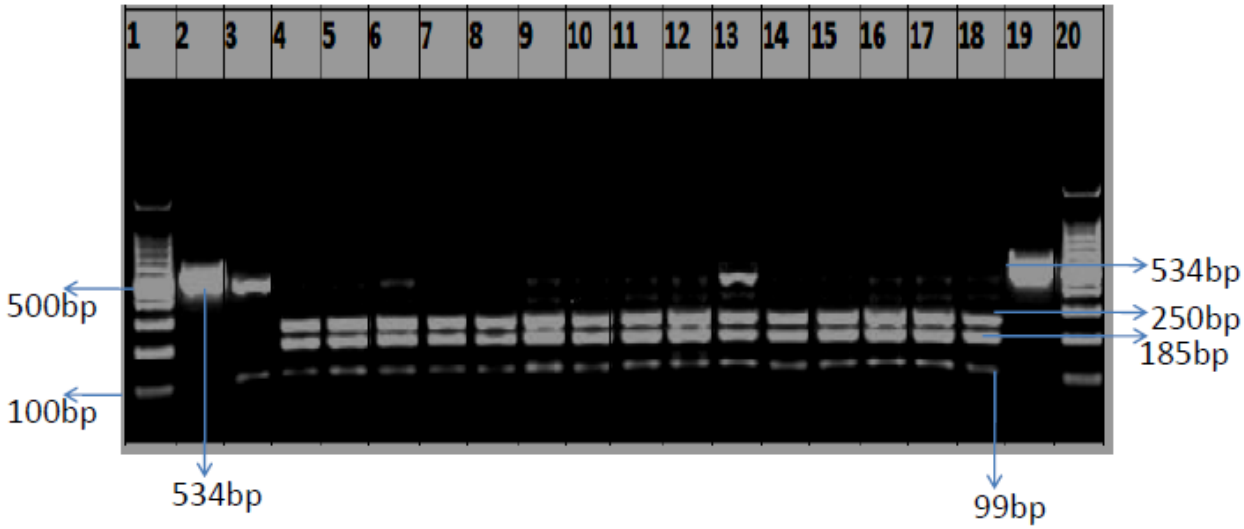


Figure 10: Representative agarose gel images for restriction analysis of the analyzed SNPs taken at AHRI lab. PCR activity from January to May, 2017: *Pf*mdr1 N86Y digestion with Apo I enzyme

(N2 product size=534BP; cleaved product size= 250+185+99 BP); lane 1-100BP standard DNA ladder, 2-undigested control, 3-18- samples, 19- undigested control, 20- 100BP standard DNA ladder.

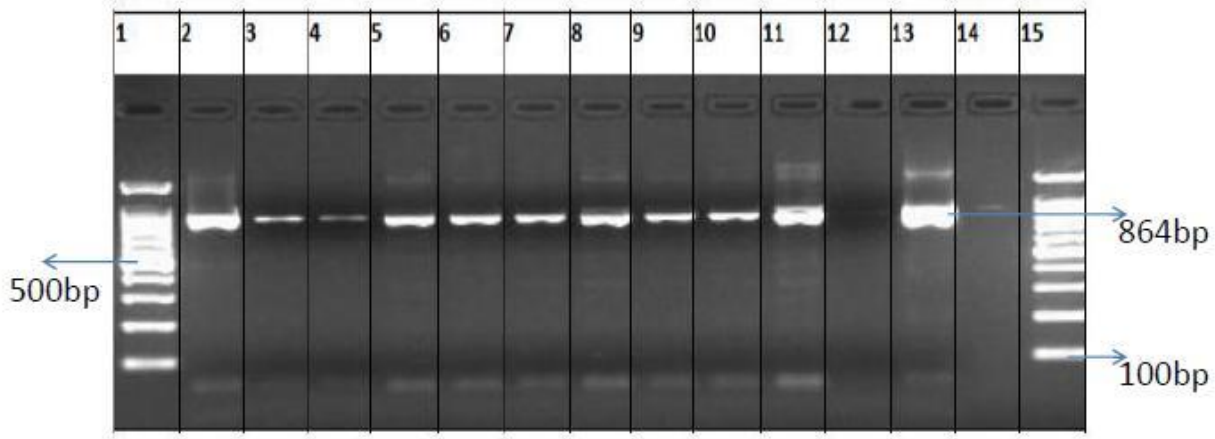


Figure 11: Representative agarose gel images for restriction analysis of the analyzed SNPs taken at AHRI lab. PCR activity from January to May, 2017: *Pf*mdr1 S1034C with Dde I enzyme (N2 product size=864BP; No digested products); 1-100 standard DNA ladder, 2- undigested control, 3-11-samples, 12- empty, 13- undigested control, 14- empty, 15- 100BP standard DNA ladder.

Table 6: Associations of the *Pf*crt K76T and *Pf*mdr-1 N86Y combined mutation. Analysis was done on samples collected from Adama Malaria Center, Gambella and Benishangul-Gumuz Regions from September to December, 2016.

*Pf*crt K76T	*Pf*mdr-1 N86Y			
	Mutant	Wild	Mixed	Total
Mutant	0	42	2	44
Wild	2	19	1	22
Mixed	0	3	3	6
Total	2	64	6	72

Pearson $X^2(4) = 19.4$, P= 0.001

3.3.1. COMPARISON OF MUTATIONS BETWEEN STUDY SITES

The prevalence of *Pf*crt K76T and *Pf*mdr-1 N86Y mutations exhibited variation between study sites (Table 7). The *Pf*crt K76T mutation was detected in 90.9% (30/33), 40.7% (11/27) and 33.3% (5/15) analyzed samples in Gambela, Adama and Benishangul-Gumuz respectively. Meanwhile; the *Pf*mdr-1 N86Y mutation was detected in only 7.1% (2/28) of samples from Gambella (Table 8), it was not detected in any of the samples from Adama and Benishangul-Gumuz.

Table 7: Distribution of the *Pf*crt K76T mutations between the study sites. Analysis was done on samples collected from Adama Malaria Center, Gambella and Benishangul-Gumuz Regions from September to December, 2016.

No	Site	*Pf*crt K76T, % (n/N)		
		Mutant	Wild	Mixed
1	Gambela	40.7 (11/27)	55.5 (15/27)	3.7 (1/27)
2	Adama	90.9 (30/33)	3.0 (1/33)	6.0 (2/33)
3	Benishangul-Gumuz	33.3 (5/15)	46.7(7/15)	20.0 (3/15)
	Total	61.3 (46/75)	30.7 (23/75)	8.0 (6/75)

Table 8: Distribution of the *Pf*mdr-1N86Ymutation between the study sites. Analysis was done on samples collected from Adama Malaria Center, Gambella and Benishangul-Gumuz Regions from September to December, 2016.

No	Site	*Pf*mdr-1 N86Y, % (n/N)		
		Mutant	Wild	Mixed
1	Gambela	7.1 (2/28)	89.3 (25/28)	3.6 (1/28)
2	Adama	0.0 (0/33)	97.0 (32/33)	3.0 (1/33)
3	Benishangul-Gumuz	0.0 (0/14)	71.4 (10/14)	28.6 (4/14)
	Total	2.7 (2/75)	89.3 (67/75)	8.0 (6/75)

4. DISCUSSION

Understanding the characteristics of the parasite populations about mutations that offer drug resistance is crucial to introduce effective treatment, control and elimination interventions. Studies conducted in this regard are very few and limited as compared with the relevance of the issue towards the problem posed by malaria, especially for countries like Ethiopia which embarked on an ambitious and extended plan; to control and eliminate malaria in different epidemiologic areas in the coming few decades.

A number of SNPs in *Pf*crt gene are documented that modulate the parasite response to CQ; more specifically *Pf*crt K76T mutation is firmly associated with CQR in *P. falciparum*. In addition; SNPs in *Pf*mdr-1 gene are also considered as complementary factor to reinforce a strong CQR phenotype. Therefore, *Pf*crtK76T mutation accompanied by different SNPs in the *Pf*mdr-1 gene will confer a stronger CQR response to the parasite.

CQ was withdrawn from the treatment regimen of malaria due to *P. falciparum* for the past twenty years. The parasite recognized the absence of CQ pressure and gradually losing its CQR alleles, probably shifted its potential to tackle the new challenge. It can also be speculated that by retaining the CQR mutations the parasite will not afford to cost for non-existing threat (Gabryszewski *et al.*, 2016). There are several research reports that repeatedly documented the reversal of CQR alleles in *P. falciparum* in malaria endemic countries around the world following the withdrawal of CQ from the treatment regimen. Studies conducted on the prevalence of CQR alleles in *P. falciparum* after the cessation of CQ usage in many malarious countries proved the slow or gradual reversal of CQR mutations from time to time (Bushman *et al.*, 2016).

In this study the species confirmation done with 18S PCR on samples collected using RDT and Microscopy confirmation showed large variations among the two commonly used diagnostic tools for routine detection of parasites in health facilities and field screening. Among the RDT confirmed *P. falciparum* mono infections during collection from Gambella and Benishangul-Gumuz together; 57.6% (57/99) were found to bear *P. falciparum*, 6.1% (4/99) *P. vivax*, 3.0% (3/99) mixed and 33.3% (33/99) were negative for both up on PCR confirmation. Meanwhile; among the Microscopy confirmed *P. falciparum* mono-infections from Adama malaria center; 68.3% (28/41) were confirmed as mono *P. falciparum*, 7.3% (3/41) *P. vivax*, 12.2% (5/41) mixed and 12.2% (5/41) were found negative during PCR confirmation. These results were consistent with other comparative evaluation studies of these tools by (Golassa *et al.*, 2013) who reported sensitivity of microscopy and RDT compared to PCR 16.5% and 24.2% respectively. Similar study conducted in Uganda and Burkina Faso documented lower sensitivity of different RDTs and Microscopy when compared to PCR to be 75.7%, 60.1% and 69.7% in Uganda and 55.8%, 42.6% and 55.8% in Burkina Faso for panPLDH/HRP2 RDT, *Pf* PLDH/pan PLDH RDT and microscopy respectively (Kyabienze *et al.*, 2016).

This lower sensitivity and detection limit observed in the commonly used diagnostic tools will inevitably leads to misclassification of infections and mistreatment which altogether favors the development, fixation and spread of anti-malarial drug resistance. In addition; the results of socio-demographic data obtained from participants that could contribute for development and spread of anti-malarial drug resistance included the habit of taking anti-malarial drugs without diagnosis; 7.3% (3/41) of participants had history of taking anti-malarial without diagnosis. As a result the parasites population faces a continual drug exposure in such co-endemic areas like Adama and the surroundings. The finding of this study also showed the fixation of the major

CQR allele, *Pf*crt K76T mutation in the co-endemic set up in 90.9% (30/33) of samples from Adama and the surroundings.

The prevalence of the *Pf*mdr-1 N86Y mutation was determined to be 2.7% (2/75) in combined analyses. The variation of the results in the two analyses using the Afl III enzyme which recognize the mutant allele (86Y) and Apo I that recognize the wild type allele (N86) may be justified with the variation in recognition sites of the two enzymes, the presence of multiple recognition sites in a target may have brought about the variation as these are the major setbacks of the PCR-RFLP technique(Rasmussen, 2012).

The findings of this study on the *Pf*crt K76T mutation are in partial agreement with the reported results by (Golassa *et al.*, 2014), who reported 100% fixation of the *Pf*crt K76T mutations in samples collected from both clinical patients and asymptomatic individuals in Shalla District, Western Arsi zone from November through December, 2012, since this study documented the *Pf*crt K76T mutation in 90.9% (30/33) of the clinical samples in investigation conducted after 4 years interval in Adama and its surroundings which is both adjacent and co-endemic setting like that of the study sites covered by the investigator.

It is also in line with the 2015 report by the same author, who showed the occurrence of the *Pf*crt K76T mutation in 72.7% of samples from Gambella almost 4 years before this investigation (Golassa *et al.*, 2015) as slow reversal is expected; this study recorded the *Pf*crt K76T mutation in 40.7% of the asymptomatic samples from Gambella. . This study also recorded the *Pf*mdr-1 N86Y mutation in 7.4 % (2/27) of the investigated samples from Gambella which is also in line with the aforementioned report who found 86Y allele in 26.1% of the clinical samples in Gambella during the study period. At the same time Golassa et al (2015), reported the occurrence of the *Pf*mdr-1 N86Y mutation in 7% and 2% of the clinical samples from Adama and West Arsi

respectively; which is in agreement with the result of this study that documented no mutant *Pf*mdr-1 N86Y allele in clinical samples from Adama and its surroundings.

Moreover, the findings of this study support the report of Mula *et al.*, (2011), who reported the 100% fixation of the *Pf*crt K76T mutation in all clinical isolates. It is also in agreement with the same authors who came up with the finding of *Pf*mdr-1 N86Y mutation or CQR allele in 32.9 % of the clinical isolates collected in different months across the period 2007 to 2009 in Gambo Rural Hospital, Southern Ethiopia.

The results of this study demonstrated about 89.3% of samples to carry the wild type *Pf*mdr-1 N86 (CQS- allele)and it is in agreement with previous report of Mekonnen *et al.*, (2014), who reported the occurrence of *Pf*mdr-1 N86 allele in 85.1% of samples collected from self-presenting febrile patients attending primary health facilities in August and December 2011 in Southern Ethiopia and in October and December 2009 in Eastern Ethiopia. However, it does not support the reported occurrence of the *Pf*crt K76T (CQS- allele) in 84.1% during the study time because, this study recorded only 30.7 % of analyzed samples from all sites to carry the wild type allele (K76), even the clinical isolates were found to be fixed (in 90.9% of the samples).

Reports on the reversal of CQR alleles from different East African countries also repeatedly confirmed the gradual reversal trends of these mutations. In Kenya a report by (Mwai *et al.*, 2009) documented the decline of the *Pf*crt K76T mutation from around 94% to 63% in 13 years time. Another study from Uganda conducted in August, 2013 to May, 2014 reported about 41.8% of samples to carry the wild type allele (K76) and the majorities (97%) of the isolates

were found to harbor the wild type (N86) allele (Ocan and Ogwal, 2016).In addition a study in Tanzania, after 10 years of CQ withdrawal from treatment regime reported the *Pf*crt K76 allele to occur in more than92% of samples (Mohammed *etal.*, 2013).A recent report also confirmed the occurrence of *Pf*crt K76 allele in 85-100 % and the *Pf*mdr-1 N86 allele in 55-97.6% of the samples collected from different study areas in Tanzania (Golassa *et al.*, 2015).Moreover; additional studies in other African countries recorded almost similar results, Equatorial Guinea, (Berzosa *et al.*, 2017) and Mauritania, (Salem *et al.*, 2017).

The result of this study showed large variations in the prevalence of CQR alleles among the study sites which have different malaria epidemiology; Gambella and Benishangul-Gumuz where *P. falciparum* dominates and Adama and the surroundings which are co-endemic for both*P. falciparum* and *P. vivax*. Moreover; this study demonstrate fixation of the *Pf*crt K76T mutation in clinical samples from Adama and the surrounding. Meanwhile; the same mutation was found at lower rate, in asymptomatic samples from Gambella and Benishangul-Gumuz respectively.

The wide difference exhibited may partly be attributed from the persisted drug pressure in the parasite populations in and around Adama because, CQ is still used for treatment of *P. vivax* malaria in these areas, 57.1% (4/7) of participants confirmed to be treated with CQ, (7.3% (3/41) of participants in this study replied to have the habit of taking anti-malarial drugs without diagnosis). In addition; there was a high difference on the parasite detection power of the three methods (Microscopy, RDT and PCR), consequently there will be misclassification and mistreatment that can expose the *P. falciparum* parasite population to CQ. Previous studies also reported high rate of misdiagnosis and misidentification in Ethiopia (Golassa, *et al.*,

2013).These and other reasons might contribute to the continual CQ pressure in the co-endemic areas. Therefore; the results of this study showed a distribution of CQR alleles that follows the drug pressure pattern.

Studies conducted in Zanzibar reported the finding of CQR related alleles in higher prevalence in asymptomatic individuals than symptomatic cases, especially the *Pf*mdr-1, (86, 184, 1246) YYY and YYD (Morris *et al.*, 2015) which are specific combinations to offer fitness against anti-malarial drugs, not defined to which specific drugs. Like the defined NFD combination which offer a fitness advantage to AL and the YFD combination which increased sensitivity to alyl-amino-alcohol drugs including mefloquine, lumefantrine, and artemisinin derivatives. However; the authors documented a general reduction of the *Pf*crt K76T and *Pf*mdr-1 N86Y mutations from 99.2%-64.7% and 89.4%-66.7% which is in line with the findings of this study.

The hypothesis of this study stated as "The prevalence of CQR alleles in *Pf* parasites has declined within the past 2 decades following the withdrawal of CQ treatment". The findings also confirmed the hypothesis and the fact that removal of CQ from treatment of *P. falciparum* malaria favored the reversal of CQR alleles. However; regardless of the percent prevalence of drug resistance alleles in asymptomatic subjects they will inevitably act as reservoir for both resistance and susceptible parasite strains and could be source of transmission and propagation hotspot as long as they are able to thrive undetected and remain untreated (Bushman *et al.*, 2016; Norahmad *et al.*, 2016).

The findings of this study confirmed the general prevalence of CQR alleles in 61.5% of samples collected along different malaria transmission areas and from asymptomatic subjects and symptomatic cases. The CQR mutations found at lower prevalence in 40.7% and 33.3% in asymptomatic samples from Gambella and Benishangul-Gumuz respectively meanwhile; fixed in 90.9% of symptomatic samples from Adama and the surroundings. The findings have

provided the existing fact about the *P. falciparum* populations in the study sites which has paramount importance with regard to the treatment, control and/or elimination programs. Moreover; the documented high reversal rate of CQR alleles will offer the opportunity to reconsider CQ, the cheapest and safest anti-malarial ever as potential treatment option in the future in areas with high reversal rates. Unfortunately, the rapid reversal of the *Pf*mdr-1 N86Y mutation and specific SNPs at different codons in other *Pf*mdr-1 gene is being associated with selection of the current ACT-based combination therapies (Thomsen*et al.*, 2013;Mbaye *et al.*, 2016) and taken as parasite preparation against the new anti-malarial drugs. The finding of this study on the combination of the two CQR alleles where, nearly complete fixation of the *Pf*crt K76T mutation and complete reversal of the *Pf*mdr 1 N86Y mutation is observed in *P. falciparum* and *P. vivax* co-endemic areas may support the suggestion that, the parasite may follow the strategy by retaining the major CQR allele *Pf*crt K76Tmutation (Sa *etal.*, 2009). Therefore, this predicted association need to be investigated to design possible intervention mechanisms to tackle the ACT resistance threat at an early stage in Ethiopia.

5. CONCLUSIONS

The following conclusions can be drawn from the findings of this study:

1. With the widely used microscopy and RDT, there was high level of misdiagnosis and misidentification of species observed compared to nPCR, which leads to mistreatment and indiscriminate use of drugs; which in turn favors the emergence and spread of drug resistance strains.
2. The rapid reversal of the *Pf*mdr-1 N86Y mutation might be attributed to the selection pressure by AL treatment.
3. Higher opportunity to re-introduce CQ for the treatment of falciparum malaria in the future especially, in Gambella and Benishangul-Gumuz Regions. However; it needs a continual assessment into the issue.

6. RECOMMENDATIONS

The following are recommendations based on the findings and gaps in knowledge identified in this study:

1. As the rapid reversal rate of the *Pf*mdr 1 N86Y mutation might be associated with selection pressure due to the current 1st line drug "ACTs (AL)" in preparation to develop resistance; this association needs immediate investigation to overcome the problem as early as possible.
2. The fixation of the major CQR determinant allele, the *Pf*crt K76T mutation in clinical isolates in co-endemic areas, suggests the continual CQ pressure in the *P. falciparum* population due to; misidentification of species, and taking CQ without diagnosis. Therefore; there should be a strong follow up and monitoring scheme on the appropriate

implementation of the treatment and diagnosis guideline at every level and on job intensive training should be given to malaria microscopists.

3. The prevalence and distribution of the major CQR-haplotypes in the country has not been fully documented, it should be investigated and documented to have the nationwide picture as input for the control effort.

7. REFERENCES

Abdul-Ghany, R., Mahdi, M. K., Beier, J., and Basco, L. K. (2017). Hidden reservoir of resistant parasites : the missing link in the elimination of falciparum malaria. *Infectious Diseases Poverty*, **6**: 12.

Alene, G. D., and Bennett, S. (1996). C hloroquine resistance of *Plasmodium falciparum* malaria in Ethiopia and Eritrea. *Tropical Medicine International Health*, **6**: 810–815.

Alifrangis, M., Dalgaard, M. B., Lusingu, J. P., Vestergaard, L. S., Staalsoe, T., *et al.*, (2006). Occurrence of the Southeast Asian / South American SVMNT haplotype of the chloroquine-resistance transporter gene in *Plasmodium falciparum* in Tanzania. *J InfectDis.*, **193**: 1738─41.

Awasthi, G., and Das, A. (2013). Genetics of chloroquine-resistant malaria : a haplotypic view. *Mems Inst Oswaldo Cruz*, **108**(8): 947–961.

Baidjoe, A., Stone, W., Ploemen, I., Shagari, S., Grignard, L., *et al.*, (2013). Combined DNA extraction and antibody elution from filter papers for the assessment of malaria transmission intensity in epidemiological studies. *Malar. J.*, **12**: 272.

Berzosa, P., Cantos, A. E., Garcia, L., Gonzalez, V., Navarro, M., *et al.*, (2017). Profile of molecular mutations in pfdhfr , pfdhps , pfmdr1 , and pfcrt genes of *Plasmodium falciparum* related to resistance to different anti- malarial drugs in the Bata District (Equatorial Guinea). *Malar. J.*, **16***:* 28.

Bloland, P. B. (2001). Drug resistance in malaria. **2000**(4):1─7. http//www.who.int/emc.

Burrows, J. N., Duparc, S., Gutteridge, W. E., Huijsduijnen, R. H. Van, Kaszubska, W., *et al.*, (2017). New developments in anti - malarial target candidate and product profiles. *Malar. Journal*, 16: 26.

Bushman, M., Morton, L., Duah, N., Quashie, N., Abuaku, B., *et al.*, (2016). Within-host competition and drug resistance in the human malaria parasite *Plasmodium falciparum*. *Proc R SocB.* **283**: 20153038.

Campbell, C. C., Collins, W. E., Teutsch, S. M., and Moss, D. M. (1979). Chloroquine resistant *Plasmodium falciparum* malaria from East Africa. *Lancet*, **314**(8153): 1151─1154.

Cowman, A. F., Healer, J., Marapana, D., and Marsh, K. (2016). Review Malaria : Biology and Disease. *Cell*, **167**(3): 610–624.

Crompton, P. D., Moebius, J., Portugal, S., Waisberg, M., Hart, G., *et al.*, (2014). Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Ann Rev Immunol.*, **32**: 157─187.

Das S., Mahapatra, S. K., Tripathy, S., Chattopadhay, S., Dash, S. K., *et al.*, (2014). Double mutation in *Pf*mdr-1 gene associates emergence of chloroqine resistant *Plasmodium falciparum* malaria in East India. *Antimicrobial Agents Chemother.*, **X**: 1─7.

Djimde, A., Kayentao, K., Doumbo, O., and Dicko, A. (2001). A molecular marker for chloroquine resistant falciparum malaria. *N Engl J Med.*, **344** (4): 257─263.

Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., *et al.*, (2009). Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med.*, **361**(5): 455–467.

Dondorp, A. M., Smithuis, F. M., Woodrow, C., and Seidlein, L. V. (2017). How to contain artemisinin- and multidrug-resistant falciparum malaria. *Trends in Parasitology*, ***xx***, 1–11.

Duah, N. O., Matrevi, S. A., Souza, D. K. De, Binnah, D. D., Tamakloe, M. M., *et al.*, (2013). Increased pfmdr1 gene copy number and the decline in pfcrt and pfmdr1 resistance alleles in Ghanaian *Plasmodium falciparum* isolates after the change of anti-malarial drug treatment policy. *Malar. J.*, **12**: 377.

Edwards, R. L., and John, A. R. (2016). Muddled mechanisms : recent progress towards antimalarial target identification. *Floo Res.*, **5**(0): 1─10.

FMOH (2004). Diagnosis and Treatment Guidelines for Health Workers in Ethiopia, Second edition. Federal Minstry of Health, Addis Ababa, Ethiopia.

FMOH (2014). National Malaria Program Monitoring and Evaluation Plan 2014-2020. Federal Minstry of Health, Addis Ababa, Ethiopia.

Fidock D. A., Nomura, T., Talley, A. K., Cooper, R. A., Sergey, M., *et al.*, (2000). Mutations in the *Plasmodium falciparum* digestive vacuole trans membrane protein *Pf*crt and evidence for their role in chloroquine resistance. *Mol Cell*, **6**(4): 861─871.

Fogh, S., Jepsen, S., and Effers, P. (1979). Chloroquine resistant *Plasmodium falciparum* malaria in Kenya. *Trop Med Hyg.*, **73**(2): 228─229.

Gabryszewski, S. J., Dhingra, S. K., Combrinck, J. M., Lewis, I. A., Callaghan, S., *et al.*, (2016). Evolution of fitness cost-neutral mutant *Pf*CRT conferring *P . falciparum* 4- aminoquinoline drug resistance is accompanied by altered parasite metabolism and digestive vacuole physiology. *PLos Pathogen*, **2**: 1–29.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., *et al.*, (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature, **419**: 6906.

Gething, P. W., Patil, A. P., Smith, D. L., Guerra, C. A., Elyazar, I. R., *et al.*, (2011). A new world malaria map: *Plasmodium falciparum* endemicity in 2010. *Malaria J.*, **10**: 378.

Golassa, L., Enweji, N., Erko, B., Aseffa, A., and Swedberg, G. (2013). Detection of a substantial number of sub- microscopic *Plasmodium falciparum* infections by polymerase chain reaction: a potential threat to malaria control and diagnosis in Ethiopi. *Malar. J.*, **12**: 352.

Golassa, L., Enweji, N., Erko, B., Aseffa, A., and Swedberg, G. (2014). High prevalence of pfcrt -CVIET haplotype in isolates from asymptomatic and symptomatic patients in south-central Oromia , Ethiopia. *Malar. J.*, **13**: 120.

Golassa, L., Kamugisha, E., Ishengoma, D. S., Baraka, V., Shayo, A., *et al.*, (2015). Identification of large variation in pfcrt , pfmdr-1 and pfubp-1 markers in *Plasmodium falciparum* isolates from Ethiopia and Tanzania. *Malar. J.*, **14**: 264.

Hailemeskel, E., Kassa, M., Taddesse, G., Mohammed, H., Woyessa, A., *et al.*, (2013). Prevalence of sulfadoxine-pyrimethamine resistance- associated mutations in *dhfr* and *dhps* genesof *Plasmodium falciparum* three years after SP withdrawal in Bahir Dar, Nortwest Ethiopia. *Acta Tropica*, **128**: 636–641.

Humphreys, G. S., Merinopoulos, I., Ahmed, J., Whitty, C. J., and Mutabingwa, T. K. (2007). Amodiaquine and artemether-lumefantrine select distinct alleles of *Plasmodium falciparum* mdr-1 gene in Tanzania children treated for uncomplicated malaria. *Antimicrobial Agents Chemother.*, **51**(3): 991–997.

Ibraheem, Z. O., Majid, R. A., Noor, S. M., Sedik, H. M., and Basir, R. (2014). Role of different *Pf* crt and *Pf* mdr-1 mutations in conferring resistance to antimalaria drugs in *Plasmodium falciparum*. *Malaria Research Traetment*, **2014:** 1–7.

Jima, D., Tesfaye, G., Medhin, A., Kebede, A., Argaw, D., and Babaniyi, O. (2005). Efficacy of sulfadoxine-pyrimethamine for the treatment of uncomplicated falciparum malaria in Ethiopia. *East African Medical J.*, **82**(8): 391–396.

Kamau, E., Campino, S., Amenga-Etego, L., Drury, E., Ishengoma, D., *et al.*, (2015). K13-propeller polymorphisms in plasmodium falciparum parasites from sub-saharan Africa. *Journal of Infectious Diseases*, **211**(8): 1352–1355.

Kyabayenze, D. J., Zongo, I., Cunningham, J., Gotton, M., Angutoko, P., *et al.*, (2016). HRP2 and pLDH-Based rapid diagnostic tests , expert microscopy , and PCR for detection of malaria infection during pregnancy and at delivery in areas of varied transmission : A prospective cohort study in Burkina Faso and Uganda. *PLoS ONE.*, **11**(7): e0156954.

Mbaye, A., Dieye, B., Ndiaye, Y. D., Bei, A. K., Muna, A., *et al.*, (2016). Selection of N86F184D1246 haplotype of Pfmrd1 gene by artemether – lumefantrine drug pressure on *Plasmodium falciparum* populations in Senegal. *Malar. J.*, **15**: 433.

Mekonnen, S. K., Aseffa, A., Berhe, N., Teklehaymanot, T., Clouse, R. M., *et al.*, (2014). Return of chloroquine-sensitive *Plasmodium falciparum* parasites and emergence of chloroquine-resistant Plasmodium vivax in Ethiopia. *Malar. J.*, **13**: 244.

Miles, A., Iqbal, Z., Vauterin, P., Pearson, R., Campino, S., *et al.*, (2016). Indels , structural variation , and recombination drive genomic diversity in *Plasmodium falciparum*. *Genome Res.*, **26**: 1288–1299.

Mohammed, A., Ndaro, A., Kalinga, A., Manjurano, A., Mosha, J. F., *et al.*, (2013). Trends in chloroquine resistance marker , Pfcrt-K76T mutation ten years after chloroquine withdrawal in Tanzania. *Malar. J.*, **12**: 415.

Morris, U., Xu, W., Msellem, M. I., Schwartz, A., Abass, A., *et al.*, (2015). Characterizing temporal trends in asymptomatic *Plasmodium* infections and transporter polymorphismsduring transition from high to low transmission in Zanzibar, 2015-2013. *Infect Genet Evol.*, **33**: 110─117.

Mula, P., Fernandez-martinez, A., Lucio, A. De, Ramos, J. M., Reyes, F., *et al.*, (2011). Detection of high levels of mutations involved in anti-malarial drug resistance in *Plasmodium falciparum* and *Plasmodium vivax* at a rural hospital in southern Ethiopia. *Malar. J.*, **10**: 214.

Mwai, L., Ochong, E., Abdirahman, A., Kiara, S. M., Ward, S., *et al.*, (2009). Chloroquine resistance before and after its withdrawal in Kenya. *Malar. J.*, **8**: 106.

Mwanza, S., Joshi, S., Nambozi, M., Chileshe, J., Malunga, P., *et al.*, (2016). The return of chloroquine- susceptible Plasmodium falciparum malaria in Zambia. *Malaria J.*, **15**: 584.

Ng, C. L., Siciliano, G., Lee, M. C. S., Almeida, M. J. De, Corey, V. C., *et al.*, (2016). CRISPR-Cas9-modified pfmdr1 protects *Plasmodium falciparum* asexual blood stages and gametocytes against a class of piperazine-containing compounds but potentiates

artemisinin-based combination therapy partner drugs. *Molecular Biol.*, **00**(00): 1–14.

Norahmad, N. A., Ridzuan, M., Abd-Razak, M., Abdullah, N. R., Sastu, R., *et al.*, (2016). Prevalence of *Plasmodium falciparum* molecular markers of antimalarial drug resistance in a residual malaria focus area in Sabah , Malaysia. *PLos ONE.*, **11**(10): e0165515.

Ocan, M., and Ogwal-okeng, J. (2016). Incomplete reversal of genotypic resistance of *Plasmodium falciparum* to chloroquine after a decade of change in malaria treatment policy in Uganda. *Reports Parasitol.*, **5**: 15–22.

Oladipo, O. O., Welington, O. A., and Sutherland, C. J. (2015). Persistence of chloroquine-resistant haplotypes of *Plasmodium falciparum* in children with uncomplicated malaria in Lagos, Nigeria, four years after change of chloroquine as first-line antimalarial medicine. *Diagn Pathol.*, **10**(1): 41.

Pholwat, S., Liu, J., Stroup, S., Jacob, S. T., Banura, P., *et al.*, (2017). The malaria Taq Man Array Card includes 87 assays for *Plasmodium falciparum* rug resistance, identification of species, and genotyping in a single reaction. *Antimicrobial Agents Chemother.*, **61**(5): e00110-17.

Price, R. N., Seidlein, L. Von, Valecha, N., Nosten, F., Baird, J. K., and White, N. J. (2014). Global extent of chloroquine-resistant *Plasmodium vivax* : a systematic review and meta-analysis. *The Lancet Infectious Diseases*, **14**(10): 982–991.

Pulcini, S., Staines, H. M., Lee, A. H., Shafik, S. H., Bouyer, G., *et al.*, (2015). Mutations in the *Plasmodium falciparum* chloroquine resistance transporter *Pf*CRT, enlarge the parasite ' s food vacuole and alter drug sensitivities. *Nature*,**5**: 14552.

Rasmussen, H. B. (2012). Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP) and Gel Electrophoresis – Valuable Tool for Genotyping and Genetic Fingerprinting. **In**: (*Gel Electrophoresis- Principles and Basics*) INTECH., Denmark, pp316–334.

Sa, J. M., Twu, O., Hayton, K., Reyes, S., Fay, M. P., et al., (2009). Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine. *Proceedings National Academy Sciences e United States America*, **106**(45): 18883–9.

Salem, M., Ahmedou, O., Lekweiry, K. M., Bouchiba, H., Pascual, A., *et al.*, (2017). Characterization of *Plasmodium falciparum* genes associated with drug resistance in Hodh

Elgharbi , a malaria hotspot near Malian – Mauritanian border. *Malaria J.*, **16**: 140.

Snounou, G., Viriyakosol, S., Zhu, X. P., Jarra, W., Pinherio, L., *et al.*, (1993). High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Molecular Biochemical Parasitol.*, 61: 315─320.

Sturrock, H. J W., Hsiang, M. S., Cohen, J. M., Smith, D. L., Greenhouse, B. *et al.*, (2013). Targeting asymptomatic malaria infctions: Active surveillance in control and elimination. *PLos Med.*, **10**(6): e1001467.

Sullivan, L. M. (2011). Power and Sample Size Determination, **In**: (*Essentials of Biostatistics in Public Health*). Boston University, School of Public Health, Boston. sphweb.bumc.bu.edu.

Tadesse, F. G., Pett, H., Baidjoe, A., Lanke, K., Grignard, L., *et al.*, (2015). 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. *Malar. J.*, **14**: 303.

Tajebe, A., Aemero, M., Francis, K., and Magoma, G. (2015). Identification of chloroquine resistance *Pf*crt K76T and detection of *Pf*mdr-1 N86Y copy number by SYBR Green I qPCR. *Asian Pacific Journal Tropical Biomedicine*, **5**(3): 208–220.

Tessema, K., Kassa, M., Kebede, A., Mohammed, H.,Leta, G., *et al.*, (2015). Declining trends of Plasmodium falciparum dihidrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) mutant alleles after the withrawal of sulfadoxine-pyrimethamine in North Western Ethiopia. *PLoS ONE.*, **10**(10): e0126943.

Thomsen, T. T., Madsen, L. B., Hansson, H. H., Toma, E. V., Charlwood, D., *et al.*, (2013). Rapid selection of *Plasmodium falciparum* chloroquine resistance transporter gene and multidrug resistance gene-1 haplotypes associated with past chloroquine and present artemether-lumefantrine use in Inhambane District , Southern Mozambique. *Am J Trop Med Hyg.*, **88**(3): 536–541.

University of Mary Land (2012). PCR and allele- specific restriction analysis (ASRA) of Pfcrt codon72, Pfmdr-1 codon 86. http://medschool.umaryland.edu/malaria/protocol/.

Valderamos, S. G., and Fidock, D. A. (2006). Transporters involved in resistance to antimalarial drugs. *Trends Pharmacol Sci.*, **27**(11): 594–601.

Wellems, T. E., Walkerijonah, A., and Panton, L. J. (1991). Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. *Proc Natl Acad Sci.*, **88**: 3382–3386.

WHO (2015). World Malaria Report 2015. WHO, Geneva, 2015.

WHO (2015). Global Technical Strategy for Malaria 2016-2030. WHO, Geneva, 2015.

WHO (2016). World Malaria Report 2016. WHO, Geneva 2016.

WHO (2016). Malaria country profiles 2016: Ethiopia. WHO, Geneva, 2016.

WHO (2016). Malaria fact sheet 2016. http://www.who.int/mediacentre/factsheet/fs094/en/.

Zhou, Z., Poe, A. C., Limor, J., Grady, K. K., Goldman, I., *et al*., (2006). Pyrosequencing , a high-throughput method for detecting single nucleotide polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes of *Plasmodium falciparum*. *J ClinicalMicrobiol*., **44**(11): 3900–3910.

8. APPENDICES

8.1. APPENDIX I: CONSENT FORMS

8.1.1. Consent form for adults above 18 years

Participant information and consent form for adults (above 18 years)

Participant information:

The prevalence of chloroquine resistance alleles in *P. falciparum* after two decades of withdrawal of chloroquine usagefor the treatment of falciparum malaria in Ethiopia.

Purpose of the study

Ethiopia has enjoyed a remarkable decrease in malaria incidence and mortality in the last decade. However, it is not clear if this success will continue and if malaria may disappear from Ethiopia or if malaria may persist. One of the challenges in malaria control and elimination is the absence or limitation of information on the current status, behaviors and new adaptation mechanisms introduced by the parasites due to fast and repeated mutations that enable them to avoid the treatment, control and elimination measures implemented in such programs. In this study, we want to determine the population structure, frequency and distribution of chloroquine resistance *Pf*crt and *Pf*mdr-1 alleles among population of *P.falciparum* and assess the possibility of reemergence of chloroquine susceptible strains in Ethiopia. In this very moment of an urgent need for alternative treatment for falciparum malaria due to resistance and failure of the currently available artemisinin and lumefantrine combination therapy is evident in many endemic countries. For this we perform studies in different areas of Ethiopia.

This study will provide highly relevant information for national malaria control and elimination effort; findings are likely to be of value outside Ethiopia. Every study comes with a discomfort for participants, we have done all the best to minimize the discomfort and all procedures will adhere to Good Clinical Practice and the Declaration of Helsinki.

We therefore ask your permission to allow us collect finger prick (5-10µl) blood sample from you. This sample will be used for future related to the spread of malaria. 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.

1) Study procedures

This information sheet will be delivered by one of the members of the study team. Completion of this form requires a maximum of 20 minutes. We would like to thank you in advance for your patience and taking part in the study.

You will be asked to donate a small finger prick blood sample that we will need for the study. Blood sample will be collected by female and male nurses from women and men respectively.

2) Voluntary participation

Your decision not to participate in this study will not affect the care you will receive in any way and you will have access to the same level of clinical care you seek from the health facility.

3) Discomfort and risks

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

4) Confidentiality statement

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

5) Long term storage of samples

We will ask your consent for long term storage of your samples, in case new techniques may be available to study the research question that we want to answer in this study. We will make samples anonymous by removing the identification information. Samples may be stored at any of

the collaborating institutions in Ethiopia, United Kingdom, Canada or The Netherlands. We will only test for factors related to study chloroquine resistance *Pf*crt and *Pf*mdr-1 allele frequency and distribution. If further studies are conducted on stored materials, ethics approval will be sought.

6) Freedom to ask questions

If you have any question concerning this trial, do not hesitate to contact the investigator of the study, Temesgen Menberu, +251911746793 Addis Ababa University or EPHI. Result from the study will be communicated to your community. In case you want to contact an independent person, not related to the study, about the research study itself, your rights as a research subject or any research related injury, you can contact the secretariat of the ethics committee at Addis Ababa University CNS, (Tel.+251116551901) and ALERT/AHRI ethics committee at (0118-962183).

8.1.2. Informed consent form for adults (above 18 years)

The prevalence of chloroquine resistance alleles in *P. falciparum* after two decades of withdrawal of chloroquine usage for the treatment of falciparum malaria in Ethiopia.

I, _____ (Participants name), having full capacity to do, hereby consent to my participation in the research study entitled, '' Assessing the prevalence and distribution of chloroquine resistance *Pf*crt and *Pf*mdr-1 alleles among *P. falciparum* population in Ethiopia.'' Under the principal investigator, Temesgen Menberu. The implications of my voluntary participation, the nature, duration and purpose; methods and means by which it is to be conducted; and the inconveniences and hazards which may reasonably be expected have been explained to me by, _____, and are set forth in the informed consent explanation. I have been given an opportunity to ask questions concerning this investigational study, and such questions have been answered to my full and complete satisfaction. If there are further questions that may arise, I may contact Temesgen Menberu Addis Ababa University CNS, or EPHI at (+251911746793) or the secretariat of the ethics committee of Addis Ababa University, CNS (+251116551901) and ALERT/AHRI at (0118-962183), an independent one. I understand that I have the right not to agree to participat e in the study.

I acknowledge / do not acknowledge the receipt of explanation on this informed consent form.

(circle one of the options)

I understand / do not understand the practical consequences of this study, asking me a finger prick blood at one occasion.

(circle one of the options)

I approve / disapprove part of the sample to be analyzed outside Ethiopia.

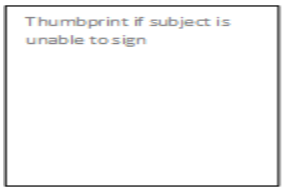
(circle one of the options)

I approve / disapprove part of the sample to be stored for future analyses, if studies are conducted using stored material, approval from ethics committee will be sought.

(circle one of the options)

I agree / disagree to be interviewed for the questionnaire. (circle one of the options)

I agree / disagree to take part in this study. (circle one of the options)



Participant's name, _____

Participant's signature, _____

Date, _____

Impartial witness's name, _____

Impartial witness's name, _____

Date, _____

Local investigator's name, _____

Local investigator's name, _____

Date, _____

8.1.3. Informed consent form for children age from 1- 17.

The prevalence of chloroquine resistance alleles in *P. falciparum* after two decades of withdrawal of chloroquine usage for the treatment of falciparum malaraia in Ethiopia.

I, _____ (Participants name), having full capacity to do, hereby consent to participation of my child, _____, (child's name) in the research study entitled, '' Assessing the prevalence and distribution of chloro quine resistance *Pf*crt and *Pf*mdr-1 alleles among *P.falciparum* population in Ethiopia." Under the principal investigator, Temesgen Menberu. The implications of my voluntary participation, the nature, duration and purpose; methods and means by which it is to be conducted; and the inconveniences and hazards which may reasonably be expected have been explained to me by, _____, and are set forth in the informed consent explanation. I have been given an opportunity to ask questions concerning this investigational study, and such questions have been answered to my full and complete satisfaction. If there are further questions that may arise, I may contact Temesgen Menberu at (+251911746793) or the secretariat of the ethics committee of Addis Ababa University CNS, (+251116551901) and ALERT/AHRI at (0118-962183), an independent one. I understand that I have the right not to agree to participate in the study.

I acknowledge / do not acknowledge the receipt of explanation on this informed consent form.

(circle one of the options)

I understand / do not understand the practical consequences of this study, asking me a finger prick blood at one occasion.

(circle one of the options)

I approve / disapprove part of the sample to be analyzed outside Ethiopia.

(circle one of the options)

I approve / disapprove part of the sample to be stored for future analyses, if studies are conducted using stored material, approval from ethics committee will be sought.

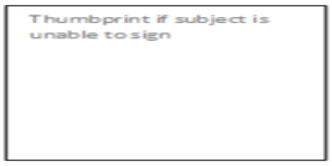
(circle one of the options)

I agree / disagree to be interviewed for the questionnaire. (circle one of the options)

I agree / disagree to take part in this study. (circle one of the options)

Participant's name, _____

Parent's / guardian's name, _____



Parent's / guardian's signature, _____

Date, _____

Impartial witness's name, _____

Impartial witness's name, _____

Date, _____

Local investigator's name, _____

Local investigator's name, _____

Date, _____

8.1.4. Oromifa translated version of consent form

UnkaWaliigalteeHirmaattootaQorannichaa

Pilaasmodiyeemfaalsiparamkanjedhaman fi dhukkubabusaakanfidanakkasumaskiloorokuwiiniinwalbaranii*Pf*CRT fi *Pf*MDR-1kan jedhamansafartuusanyiifayyadamuunItiyoophiyaattihaalairrajiruu fi tamsasinaisaaakkasumasjijjiiramainniagarsiiseqorachuu.

Ani _____ (maqaahirmaataan), matadureeqorannoo "Pilaasmodiyeemfaalsiparamkanjedhaman fi dhukkubabusaakanfidanakkasumaskiloorokuwiiniinwalbaranii*Pf*CRT fi *Pf*MDR-1kan jedhamansafartuusanyiifayyadamuunItiyoophiyaattihaalairrajiruu fi tamsasinaisaaakkasumasjijjiiramainniagarsiiseqorachuu" isajedhugaggeessaqorannichaaobboTamasgeenManbaruutiingaggeefamuhirmaahuufwaliigaleera. Kaayyooqorannichaa, haala, yeroo, qorannichagaggeessuuftoftaale fi mala akkasumashir'inootamul'achuudanda'anibsa _____tiineddanaaftaasifameeboodahubadheewaliigaleera.Wa'eeqorannichaagaafachuufcarraannaafkennameera.Gaaffileengaafadheefisdeebiinisaaniiargadheera.Gaaffileedabalataayoonqabaadheobbo Tamasgeen Manbaruutiinlakk. +251911746793 irrattiargachuunindanda'ayknHoospitaalaAlartii fi "Armauer Hansen Research Institute (AHRI)" ttikanargamanbarreessaakoreeraawwiikanta'ansararabilbilaa0118-962183 tiinargachuunindanda'a.

Fedhiikooqofairrattikanhunda'emaleedhibbaaeenyuunlleeakkahintaanehubadheera.

Waa'eeqorannichaaibsaargachuukoo :Argadheera/ Hinarganne(ittimaraa):

Yerootokkoqobaharkaakooxiqqoowaraanuundhiigafudhatamu fi waa'eebu'aaqorannicha:Hubadheera/HinHubanne(ittimaraa).

Itoophiyaatiin ala saampilootamuraasairrattiqorannoonakkagaggeeffamu: Waliigaleera/ Waliihingalle(ittimaraa).

Saampilootniqorannaafsassaabamanmuraasniyeroomuraasaafakkaolka'amuu fi qorannoondabalataaakkagaggeeffamu:waliigaleera/ waliihingalle(ittimaraa).Yooqorannoodabalataasaampilootaka'amanirrattikangaggeefamanta'eee yyamnikoreenraawwiikangaafatamuta'a.

Gaafanooqorannichaaguutuuf, gafannooafaaniigochuuf :Waliigaleera/ waliihingalle.
Qorannichairrattihirmaachuuf: Waliigaleera/ waliihingalle.

Warrabarreesuuhindandeegneemallatooquba as haaka'an

Maatii/ Gudistoota

MaqaaHirmaataa: _____

Guyyaa: _____

Maqaaragaalee:_____

Mallattooragaaalee :-_____Guyyaa:- _____

Maqaaqoratichaa : _____
Mallattooqoratichaa : _____ Guyyaa: _____

8.1.5. Amharic translated version of consent form

የ ጥና ቱተሳ ታፊየ ስ ምምን ትመገ ለ ጭቋ ጽ

ክ ሎሮኪ ን የ ተ ለ ሞ ም ዱ ፕ ላ ስ ሞ ዲ ዬ ም ፋ ል ሲ ፓ ረ ም የ ተ ባ ሉ የ ወ ባ አ ም ጬ ፓ ራ ሳ ይ ት ዝ ር ያ ዎ ች ን የ *Pf* CRT እ ና *Pf* MDR-

1የ ተ ባ ሉ የ ዘ ረ ሜ ል ማ ላ ኪ ያ ዎ ች ን በ ማ ከ ቀ ም ባ ኢ ት ዮ ጵ ያ ያ ላ ቸ ዉ ን ክ ስ ተ ት ማ ከ ን ና ስ ር ጭ ት እ ን ዲ ሁ ም ዋ ነ ኛ ል ዉ ጥ ዓ ይ ነ ቶ ች ን ማ ጥ ና ት።

እ ኔ _____(የ ጥና ቱተሳ ታፊስ ም),

በ አ ር እ ስ ቱ በ ተ ጠ ቀ ሰ ዉ ብ ዋ ና ተ ሚ ሚ ዉ ተ ማ ከ ገ ን መ ን በ ሩ ከ በ ደ የ ሚ ሚ ዉ ጥ ና ት ላ ይ እ ን ድ ሳ ተ ፍ ተ ስ ማ ም ቻ ለ ሁ፡-

“ክ ሎ ሮ ኪ ን የ ተ ለ ሞ ም ዱ ፕ ላ ስ ሞ ዲ ዬ ም ፋ ል ሲ ፓ ረ ም ሞ ተ ባ ሉ የ ወ ባ አ ም ጬ ፓ ራ ሳ ይ ት ዝ ር ያ ዎ ች ን የ *Pf* CRT እ ና *Pf* MDR-1

የ ተ ባ ሉ የ ዘ ረ ሜ ል ማ ላ ኪ ያ ዎ ች ን በ ማ ከ ቀ ም ባ ኢ ት ዮ ጵ ያ ያ ላ ቸ ዉ ን ክ ስ ተ ት ማ ከ ን ና ስ ር ጭ ት እ ን ዲ ሁ ም ሞ ነ ኛ ል ዉ ጥ ዓ ይ ነ ቶ ች ን ማ ጥ ና ት

። “ሥ ለ ጥና ቱ ዓ ላ ማ ፣ ሁ ኔ ታ ፣ ጊ ዜ ፣ ጥና ቱ ን ለ ማ ከ ራ ት የ ሚ ከ ተ ል ሙ ት ን ዘ ዴ ዎ ች ና ብ ል ሃ ቶ ች ን ሊ ከ ሰ ቱ የ ሚ ች ሉ ት ን አ ለ መ መ ቶ ች ተ ረ ድ

ቼ በ

ገ ለ ጻ ከ ተ ደ ረ ገ ል ኝ በ ሁ ዋ ላ ተ ስ ማ ም ቻ ለ ሁ። ፡ ስ ለ ጥና ቱ የ ማ ከ የ ቅ እ ድ ል ተ ሰ ጥ ቶ ኛ ል ፤ ለ ጠ የ ኩ ዋ ች ዉ ም ጥ

ያ ቄ ዎ ች ተ ገ ቢ ዉ ን ም ላ ሽ አ ግ ኝ ቻ ለ ሁ። ፡ ተ ጨ ማ ሪ ጥ ያ ቄ ዎ ች ቢ ኖ ሩ ኝ ተ ማ ከ ገ ን መ ን በ ሩ ን በ ስ ል ክ ቁ ጥ ር +251911746793

ማ ግ ኘ ት እ ች ላ ለ ሁ ወ ይ ም የ አ ለ ር ት ሆ ስ ፒ ታ ል እ ና የ አ ር ሜ ር ሃ ን ሰ ን ም ር ም ር ማ እ ከ ል የ ስ ነ - ም ግ ባ ር ኮ ሜ ጸ ሃ ፊ ን በ ተ ማ ላ ከ ተ ዉ አ ድ ራ ሻ ማ ግ ኘ ት ይ ች ላ ሉ(0118962183)። ፡ በ ራ ሴ ፈ ቃ ድ ና ፍ ላ ጎ ት ካ ል ሆ ነ በ ቀ ር በ ሌ ሎ ች ጩ በ ጥና ቱ ማ ሳ ተ ፍ የ ሜ ል ገ ደ ድ ና የ ማ ይ ገ ባ ማ ሆ ኑ ን እ ገ ነ ዘ ባ ለ ሁ። ፡

አ ግ ኝ ቻ ለ ሁ / አ ላ ገ ኝ ሁ ም ፤ ስ ለ ጥና ቱ ተ ገ ቢ ዉ ን መ ገ ለ ጩ ማ ግ ኘ ቴ ን (ያ ክ ብ ቡ)

ተ ረ ድ ቻ ለ ሁ /
አ ል ተ ረ ዳ ሁ ም ፤ ለ አ ን ድ ጊ ዜ ብ ቻ ጣ ቴ ን በ ማ ቡ ጣ ት ስ ለ ሚ ወ ሰ ድ ደ ማ ከ በ ታ ና ሞ ኖ ስ ለ ጥና ቱ ተ ጨ ባ ጭ ዉ ኬ ት

(ያ ክ ብ ቡ)

ተ ስ ማ ም ቻ ለ ሁ / አ ል ተ ስ ማ ማ ሁ ም ፤ የ ተ ወ ሰ ነ ና ሞ ኖ ከ ኢ ት ዮ ጵ ያ ዉ ጪ ሞ ር ም እ ን ዲ ደ ረ ግ በ ት (ያ ክ ብ ቡ)

ተስማምቻለሁ /
አልተስማማሁም፤ የተወሰነ ና ማኖ ለተወሰነ ጊዜእን ዲቀጭና ተጨማሪ ምር ምር እን ዲደረግበት

(ያክብቡ)። ተጨማሪ ምር ምሮች በተቀጣጭና ማኖ ዎች ላይየ ሚሰራከሆነ የ ስነ ምግባር ኮ ሜቴን ይሁን ታ የ ሚከየ ቅበ ትይሆና ል፡ ፡

ተስማምቻለሁ / አልተስማማሁም፤ ማከይቁን ለመጫ ትየ ቃል ጥያ ቄ ለሚድረግ (ያክብቡ)
ተስማምቻለሁ / አልተስማማሁም፤ በጥና ቱለ ማሳ ተፌ
(ያክብቡ)

መፃ ፍለ ሚይችሉየ ጥትእሻ ራአ ዚህያ ሳርፉ ወላጅ/ያሳዳጊ

የተሳታፊስም-_____

ቀን፡-_____

የገለልተኛ ምክክር ስም- _____
የገለልተኛ ምክክር ፊርማ- _____ ቀን፡- _____

የተሟሚ ውዋ ሰም- _____
የተሟሚ ውዋ ፊርማ- _____ ቀን፡- _____

8.2. APPENDIX II: QUESTIONNAIRE-BASED INTERVIEW

Title of the study: The prevalence of chloroquine resistance alleles in *P. falciparum* after two decades of withdrawal of chloroqine usage for the treatment of falciparum malaria in Ethiopia.

The paper based interview will be filed in a questionnaire and data will be collected by the interviewers. The interviewer begins by introducing himself and the purpose of the study to the participants, requesting the respondents for participation in the survey, thanking the participant for giving consent to take part in the study.

Date, __ __ /__ __/ ___ ____ ___ ____.(DD/MM/YYYY).

1. Participant's name: _____
2. Participant's code: _ __ / __ __ __/__ __ _____ ___
3. Participant's telephone number: __ __ ____ ___ ___ ___
4. Date of birth: ___ ___/___ ___/___ ___ ___ ___(DD/MM/YYYY)
5. Age (in years): _____
6. Gender: Male_____, Female_____
7. Woreda (District) name: _____
8. Name of town or village: _____
9. Household number: _____
10. What is the nearest health facility to your house?
    a) Hospital b) Health center
    c) Clinic d) Health post

11. how far is the nearest health institution located (time it takes in minute, to arrive there with the available transportation)? _____ minutes.

12. have you (has the child) had malaria in the last three weeks?

    a) Yes b) No c) Don't know

13. if yes, when exactly, _____

14. was treatment given?

a) Yes, name of the drug if known, _____

b) No,

c) Don't know

15. Parent/guardian name (for children <18 years old): _____

16. What kind of water bodies exists in your neighborhood? (you can give more than one answer)

a) None c) Lake e) Swamp

b) River d) Pond f) Stagnant water

g) Other (specify), _____

17. How far (in minute) is the nearest water body located from your house on walking?

_____ minutes.

18. Does your household have any of the following?

a) Electricity:

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

b) Radio:

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

c) Television:

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

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

a) Grass thatch c) wood and mud

b) Iron sheet d) Other, _____

20. 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

21. What is the main material of the house's floor?

a) Earth d) Wood

b) Local dung plaster e) Other, _____

c) Cement

22. Are the eaves open or closed?

a) Open c) Partially open

b) Closed d) No eaves/not applicable

23. Do you own a bed net for yourself (the child in the study)?

a) Yes b) No c) Don't know

24. Do you use the bed net (the child in the study during the last night?

a) Yes b) No c) Don't know

25. How many people permanently live in the house? _____

26. How many bed nets are available in the house? _____

27. At any time in the past 12 months, have the interior wall of the house been sprayed against mosquitoes?

a) Yes b) No c) Don't know

28. How many months ago was the house sprayed? (If less than one month, record '00' months ago)? _____

29. Have you (your child) made any overnight trips outside of (the District) in the last 3 months?

a) Yes b) No c) Don't know

30. If yes, where did you (your child) spend the most time during this overnight trip and when exactly? _____

31. What time you (does the child) enter the house usually in the night? _____

32. What time did you (the child) enter the sleeping space last night? _____

33. What time do you (the child) leave the sleeping space in the morning? _____

34. What time do you (the child) leave the house in the morning? _____

35. Temperature (^{o}C), _____

If ≥ 37.5 ^{o}C carry out RDT

If < 37.5 ^{o}C skip to Hb results

36. RDT taken?

a) Yes b) No

37.If not, why not? _____

38. RDT result?

a) Positive b) Negative c) Invalid

If positive, refer to the nearest health institution for treatment.

39. Hb taken?

a) Yes b) No

40.If not, why not? _____

41. Hb result? _____

If, less than 8g/dl, refer to the nearest health institution for treatment.

42. Microtainer sample taken?

a) Yes b) No

43.If not, why not? _____

44. Microscope slide film taken?

a) Yes b) No

45.If not, why not? _____

46. Filter paper taken?

a) Yes b) No

47.If not, why not? _____