DETECTION OF *Plasmodium falciparum* AND *P. vivax* MIXED INFECTION LEVEL AND IDENTIFICATION OF *Pfcrt* AND *Pfmdr1* RESISTANCE MUTATIONS IN NORTH-WEST ETHIOPIA

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Detection of *Plasmodium falciparum* and *P. vivax* Mixed Infection Level and Identification of *Pfcrt* and *Pfmdr1* Resistance Mutations in North-West Ethiopia

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DECLARATION

This thesis is my original work and has not been presented for a degree awardin any other university.

Signature: ..... Date: .....

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This thesis has been submitted for examination with our approval as university supervisors.

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DEDICATION

The thesis is dedicated to my humble family who tirelessly helped me achieve the whole work.

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LIST OFACRONYMS

ACT	Artemethesinin Combination Therapy
AL	Artemether Lumefantrine
AQ	Amidoquine
CQR	Chloroquine Resistance
C_t	Detection threshold
CVIET	Cysteine, Isoleusine,Valanine, Glutamic Acid and Threonine
CVMNK	Cysteine,Valanine,Methionine,Asparagine and Lysine
DV	Digestive Vacuole
IC_{50}	50% Inhibitory Concentration
LU	Lumefantrine
MF	Mefloquinine
NTC	No Template Control
PFCRT	*Plasmodium falciparum* Chloroquine Resistance Transporter
PFMDR	*Plasmodium falciparum* Multidrug Resistance
RDT	Rapid Diagnostic Test

SNP	Single Nucleotide Polymorphism
SVMNT	Serine,Valanine,Methionine,Threonine and Asparagine
Tm	Melting Temperature

ABSTRACT

This study was conducted to assess the level of mixed infections and prevalence of chloroquine transporter gene point mutations that are important determinants of drug resistance level in *P. falciparum*. A total of 7,343 patients were diagnosed for malaria and the level of mixed infections of *P. falciparum* and *P. vivax* was also determinedmicroscopically. Dried blood spots were prepared from 168 positive samples and parasite DNA extracted by commercial kit and used for real-time PCR analysis. Out of the total 7, 343 samples 1,802 (24.54%) microscopically positive for *plasmodium*, (67.48%) were *P. falciparum*, (30.68%) *P. vivax* and (1.8%) mixed infection of both species. Among 168 positive samples that were selected for further analysis, 7 (4.17%) were *P. vivax*, 158 (94.05%) were *P. falciparum* and 3 (1.80%) were mixed infection of both species. The real-time PCR analysis of these 168 positive samples showed that 21 (12.50%) were *P. falciparum* and *P. vivax* mixed infections, 17 (10.12%) were *P. ovale*, 10 (5.95%) were *P. vivax* and 112 (66.67%) were *P. falciparum.* The genotyping of real-time PCR*P. falciparum* positive samples for (*Pfcrt*, K76T), (*Pfmdr1*, N86Y) and copy number variation showed (54.88%) were confirmed as mutant (*Pfcrt, 76T*) and the rest (45.11%) were wild type (*Pfcrt, K76*). The analysis of (*Pfmdr1, N86Y)* showed (45.86%) had single copy and the rest (54.13%) had multi-copy higher than 1.5 copies per genome. The results indicated (27.38%) difference and disagreement between microscopy and real- time PCR for mixed species detection and misdiagnosis. The present study showed a high prevalence and fixation of *(Pfcrt, 76T)* mutations 10 years after chloroquine withdrawal, but statistically insignificant (P=0.635, P>0.05)**.** A few samples showed

mutant *Pfmdr1,* butprevalence of *Pfmdr1* copy number variants was high suggesting the presence of inducing factors other than chloroquine for emergence of *P. falciparum* strains with higher copy numbers. However, Prevalence of (*Pfmdr1*, N86Y) copy number variant was not statistically significant (P= 0.455, P>0.05). This study recommends regular clinical diagnosis quality control and molecular surveillances on drug resistance malaria. The use of chloroquine for *P. vivax* treatment under poor diagnosis facilities should be discontinued.

Key words: Mixed infections, *Plasmodium falciparum, P. vivax,* microscopic diagnosis, real-time PCR.

CHAPTER ONE

INTRODUCTION

1.1.Background

Malaria, together with other infectious diseases such as tuberculosis and HIV, is an important cause of morbidity and mortality. Malaria is caused by the parasite of the genus *Plasmodium*, and is transmitted by anopheline mosquitoes. *Plasmodium* parasite has five species affecting human. These are *Plasmodium falciparum*, *P. vivax,P. ovale, P. malariae*, and *P. knowlesi*. These species cause approximately 225 million infections and nearly one million deaths per year globally. Among these, *P. falciparum* is the most prevalent and common malaria species worldwide, especially in Africa. It causes the most severe form of the disease and is responsible for over 90% of the malaria patients' death (Petersen *et al.*, 2011).

Malaria transmission in Ethiopia is bi-annual and the major epidemics occur every five to eight years with focal epidemics as the commonest form. The overall trend is one of a gradual progression from around four-fifths of a million confirmed cases a year in 1990 to almost a million and a half in the year 2005-06 reporting period. This represents an increase of about 80 percent (www.EthioDemographyAndHealth.Org).Malaria occurs in Ethiopia sporadically at high altitudes, and its prevalence increases towards the lowlands. It occurs throughout the year, but predominantly before and after the main rainy season September to October and April to May (Woyessa *et al*., 2012).

Plasmodium falciparum has a daunting potential for drug resistance development for any drug introduction to treat malaria. The parasite evolves to a new evolutionary line for better adaptation to anti -malaria drugs. Combination drugs are effective for treating chloroquine resistance *P. falciparum* malaria, but the parasite could develop resistance including to combination regimens with or without Artemisinin (Mharakurwa *et al*., 2011). Drug resistance malaria is a major challenge to the control of *P.falciparum* malaria in developing and low income countries particularly sub-Saharan Africa. It is the leading cause of morbidity and mortality (Atroosh *et al*., 2012).

Chloroquine, primaquine and quinine were the most popular anti-malarial drugs for malaria treatment in Ethiopia before 1998. The Federal Ministry of Health, Ethiopia, (2004a:7) recommended the use of quinine tablets and injection to treat severe malaria. The widespread chloroquine treatment failure in *P. falciparum* infections in 1997–1998 led to the introduction of Sulfadoxine/Pyrimethamine for the treatment of *P. falciparum*malaria. sulfadoxine/pyrimethamine has been in use until the adoption of coartem in 2004 (Ambachew, 2012).

After the banning of sulfadoxine/pyrimethamine, coartem is recommended to treat complicated malaria and mixed infections. Since 2005 up to date chloroquine, artesunate, quinine and coartem are in use to treat malaria (Alemu *et al*., 2012; Ambachew, 2012). Chloroquine is still in use to treat uncomplicated malaria particularly *P. vivax* because there is no enough data on treatment failure and/or chloroquine resistance to guarantee a change (Alemu *et al*., 2012, Ambachew,

2012).In the study area *P. falciparum* is common *Plasmodium* species and self-treatment is also common, so there is a danger that parasites may develop resistance to coartem and chloroquine (Alemu *et al*., 2012).

1.2.Statement of the problem

The treatment of malaria in Ethiopia is done by chloroquine and coartem nationwide. Coartem is given for complicated malaria and chloroquine is given for uncomplicated malaria especially for *P. vivax* infection. The treatment of *P. vivax* by using chloroquine as first line drug might increase the prevalence of chloroquine transporter gene point mutation and *P. falciparum* multi-drug resistance. Chloroquine is incompletely withdrawn, for 10 years, from the study area hence the (*Pfcrt*, K76T) and (*Pfmdr1*, N86Y) mutation would have been fixed in *P. falciparum* population. The prevalence of chloroquine resistance molecular markers for *P. falciparum* has not been established in the study area. Furthermore, sub-standard method of *Plasmodium* species detection does not guarantee accurate *Plasmodia* species discrimination for proper treatment regimen. Identification of morphologically similar Plasmodia species is challenging in the study area due to poor facilities and lack of regular training for technicians. Molecular based diagnosis of *Plasmodia* species is not common for clinical application.

Plasmodia species appear as mixed infections in malaria-endemic areas. When these species occurred simultaneously the species are misdiagnosed. The misdiagnosed *P. falciparum* may have an exposure to chloroquine. In contrast *P. vivax* may have also an exposure for coartem due to mixed infection and/or misdiagnosis. This situation

may impair the effectiveness of coartem and chloroquine which are treatment drugs for *P. falciparum* and *P. vivax* respectively. This phenomenon might fix previous mutations in the study area.

Self-treatment could also cause *P. falciparum* to development resistance and/or fixation of previous resistant *P. falciparum* population. Most people in the study area are not aware of drug resistance malaria species; therefore, they purchase any drug from the market without any clinical confirmatory test and use it for self- treatment (Alemu *et al*., 2012).

The long acting drugs such as chloroquine and artemisinin partner drug lumefantrine could play pivotal role in fixation and development of new resistance because long acting drugs have greater selective pressure than short acting drugs in resistance development or drug adaptation by the parasite due to long exposure time of such drugs with long elimination time (Petersen *et al.,* 2011; Cormican, 2006).

In a geographical area when more than one species coexist, sympatric combination of these infections in an individual cannot be ruled out (Mohapatra, 2012). In unstable malaria where there is seasonal variation, presumptive treatment of uncomplicated fever with anti-malarial drugs result in potentially a high proportion of misdiagnosis and consequence of mismanagement during the low malaria transmission seasons (Mitku *et al*., 2003).

Coartem is a fixed-dose combination of artemether and lumefantrine (AL), which acts as a blood schizontocide. It is indicated for treatment; including stand-by emergency

treatment of adults, children and infants with acute, uncomplicated infections due to *Plasmodium falciparum*or mixed infections. Because coartem is effective against both chloroquine sensitive and resistant *P. falciparum,*it is also recommended for malaria infections acquired in areas where parasites are resistant to chloroquine (www.coartem.com/downloads/IPL-Coartem.pdf).

Coartem is not indicated for the treatment of malaria due to *P. vivax, P. malariae* or*P. ovale* except mixed infections with *P. falciparum*. However, (Yohannes *et al*, 2011) confirmed significantly higher cumulative incidence of treatment failure in *P. vivax* cases treated with artemether and lumefantrine combination drug than with chloroquine. This efficacy study was the first in Ethiopia which has been done for the treatment of *P. vivax.Plasmodium vivax* resistance reporting from Ethiopia is quite recent and rare. There are no studies done on treatment failure in *P. vivax* cases treated with coartem globally.

Since coartem is effective against both chloroquine resistant and sensitive *P. falciparum* the prevalence level of chloroquine resistant *P. falciparum* expected as lower /or no fixation of previous mutations where coartem is used for treatment of *P. falciparum* and mixed species infection of malaria. Even though, coartem is effective against both sensitive and resistant *P. falciparum* its efficacy could be impaired due to several factors such as dose, self-treatment, and *Pfmdr1* copy number variations.

Owing to these problems the current study was conducted to establish mixed infection level and misdiagnosis. It also determined the prevalence level and copy number variations in chloroquine resistance mutations. Among all the above factors,

chloroquine resistance point mutation at (*Pfcrt, K76T)* and (*Pfmdr1, N86Y)* genes were studied as critical factors for future malaria treatment.

1.3. Justification of the study

This study was carried out to fill the knowledge gap that in malaria-endemic area of Ethiopia drug resistance development due to mixed infection and mistreatment is not well documented. The treatment of mixed infection of malaria especially *P. vivax* and *P. falciparum* is critical. The co-occurrence of the two species is difficult to manage malaria epidemics and risk of drug resistance development. Therefore, the study was greatly important to uncover point mutation prevalence and chloroquine resistance level as well as copy number variation for the first time in the study area. The study was done by detecting mixed infection level, identifying prevalence of chloroquine resistance transporter gene point mutation at (*Pfcrt,* K76T) and determining the prevalence level and copy number of (*Pfmdr1,* N86Y) mutation. Since *P. falciparum* is the most severe and the two types of mutations are strongly associated with chloroquine resistant *P.falciparum* the study focused on *P.falciparum* mutation other than on *P. vivax.*

1.4.Significance of the study

The result of this research will help the national malaria control strategy by providing valuable information on the prevalence level of mixed infections and drug resistance *P. falciparum*. Therefore, it will help the policy makers to decide on continuation or discontinuation of chloroquine monotherapy for treatment of *P. vivax* at national

level. Since the study was conducted on chloroquine resistant *P. falciparum*, it indirectly shows the negative impact of incomplete withdrawal of chloroquine monotherapy for uncomplicated malaria on fixation of previous point mutations in *P. falciparum* malaria. As a result, the study can create awareness on the fixation of chloroquine resistant *P. falciparum* population in the study area. The fixations of previous mutations affect, on the other hand, the re-introduction of chloroquine monotherapy for *P. falciparum* treatment after a long period of chloroquine withdrawal. The results indicated the need of molecular surveillance to monitor drug resistance malaria emergence and prevalence. Therefore, prophylaxis and treatment drugs could be assessed regularly. It is very important to understand the current status of drug resistance *P. falciparum*prevalence at molecular level. The prevalence level of the resistant parasite is a warning sign that the parasite could adapt even to the combination drug therapy especially the combination of chloroquine, mefloquinine, artesunate, lumefantrine and other related drugs. The study showed a high level of *P. falciparum* and *P. vivax* mixed infection and misdiagnosed malaria species in the study area. It also provided basic information for other researchers about drug resistance development risk and fixation of previous resistance mutations.

1.5. Purpose of the study

This study was conducted to detect *P. vivax* and *P. falciparum*mixed infection level, and determine the prevalence level of Chloroquine resistance *P. falciparum* in North Gondar, north-west Ethiopia.

1.5.1. Specific objectives:

1. To assess *P. vivax* and *P. falciparum* mixed infection level in febrile patients
2. To determine the prevalence of (*Pfcrt*,K76T) point mutations in *P. falciparum* isolates
3. To determine prevalence level and copy number of (*Pfmdr*1,N86Y) in *P. falciparum*isolates

CHAPTER TWO

LITERATURE REVIEW

2.1.Background

Plasmodium falciparum and *Plasmodium vivax* are the main causative agents of human malaria accounting for 300-500 million cases and 130-145 million infections per annum, respectively (Buppan *et al*., 2010). *Plasmodium falciparum*chloroquine resistance is a major cause of worldwide increases in malaria mortality and morbidity. Resistance to anti-malarial drugs is developed by structural change on membrane protein coding gene due to mutations. The *Pfcrt* protein is localized to the digestive vacuole membrane and contains 10 putative trans-membrane domains. Point mutations in *Pfcrt,* in Thr (K76T) mutation in the first predicted trans-membrane domain, show an association with chloroquine resistance in both field isolates and clinical studies (Sidhu *et al.,* 2002).

2.2.*Plasmodium* species detection

2.2.1. Diagnosis

Clinical diagnosis by microscopy is not precise, but still the basis of therapeutic care. Microscopy plays a key role in diagnosis of malaria febrile patients in developing countries. Reasonable malaria diagnosis and treatment is essential to avoid non-target effects and to save cost on alternative drugs**.** Accurate and effective diagnosis is the only way of assuring rational treatment and therapy(Wongsrichanalai *et al*., 2007).

Microscopic observation of *P. falciparum* infection is influenced by its parasite density. Parasite density of non-*falciparumPlasmodium* species infections is usually low compared to *P. falciparum* (Obare *et al*., 2013).Therefore, other *Plasmodium* species are easily missed. . Moreover, in mixed infections, the background of large numbers of *P. falciparum*parasites makes the observation difficult to differentiate other species(Fançony *et al*., 2012).

2.2.2. Co-infections

Mixed species infections can not only complicate diagnosis but also alter the severity of malaria.. For instance, *P. vivax* has been reported to have a protective effect to the severe of *P. falciparum* infections (Mohapatra *et al*., 2012)**.** The co-existence of *P. falciparum* and *P. vivax* in Ethiopia and the different levels of effectiveness of the anti-malarial drugs against the malaria parasite species demand administration of the right drug to control morbidity and mortality. In the Ethiopian setting, therefore, the current ongoing efforts to increase access to diagnostic services, including the use of appropriate rapid diagnostic tests (RDTs) are expected to have a significant contribution (Yohannes *etal*., 2011).Laboratories in malaria-endemic area needs accurate and precise diagnosis of mono-infection and mixed species infections in order to assure proper treatment decision. It helps to prevent the advent of drug resistant parasite population. In malaria-endemic areas of Ethiopia *P. falciparum* and *P. vivax* mixed infections occur with a high frequency (Mula *et al*., 2011). However, mixed infection incidence is less than the prevalence of the individual species (Mohapatra *et al*., 2012).

2.2.3 Incidence of co-infection

Mixed malaria infections are seasonal and occur more in wet seasons. The spread of high populations of various mosquito species, which have species-specific transmission, affect the prevalence of single or mixed infections. An anopheles mosquito can harbour both *P. falciparum* and *P. vivax*. Therefore, a bite of this mosquito may inoculate both species simultaneously (Mohapatra *et al*., 2012). The co-existence of both *P. falciparum* and *P. vivax* in a single human host suppress each other. *Plasmodium falciparum*suppresses *Plasmodium vivax*parasitaemia by interspecies inhibition.

Severe malaria, in mixed infection, depends on whether it is *P. falciparum* or *P. vivax* super infection. *P. vivax* infection over an existing *P. falciparum* infection leads to the rise of *P. falciparum* parasitaemia. In contrast, *P. falciparum* infection over an existing *P. vivax* infection reduces *P. falciparum* parasitaemia**.** Therefore, it prevents the development of severe malaria (Mohapatra *et al*., 2012).

If mixed infection is misdiagnosed as *P. vivax*single infection, treatment of *P. vivax*increases *P. falciparum*parasitaemia. High rates of *P. vivax*infection occur after treatment of *P. falciparum*single infection. Mixed infections aggravate the emergence of anti-malarial drug resistance. Hence, a drug-resistant population of *plasmodium*parasites increases (Lee *et al*., 2011). Therefore, accurate diagnosis or species identification of mixed infection is critical for therapeutic decision (Lee *et al*., 2011; Obare *et al*., 2013). It helps to manage the selection, dose, and timing of anti-

malarial drugs. Mistreatment of a single or multiple species have serious clinical consequences.

2.3. Chloroquine resistance *P. falciparum*

2.3.1. Chloroquine mechanism of action

Structurally chloroquine is a 4-aminoquinoline which was introduced in the late 1940s. Chloroquine was used on a massive scale for malaria treatment and prophylaxis. Its efficacy, affordability and safety, even during pregnancy, made chloroquine the standard treatment drug of malaria for a long time (Keen *et al*., 2007; Petersen *et al*., 2011).It is one of the cheapest and safest drugs ever used for malaria treatment (Cormican, 2006). It has one of the longest half-lives among anti-malaria with approximately 60 days. It provides a chemo-prophylactic effect during the drug elimination phase. It also exposes the parasites to an extended time period. As chloroquine falls below the therapeutic concentration, it may select for drug-resistant parasite populations (Petersen *et al*., 2011)

The heme detoxification pathway is chloroquine's principal target in the parasites digestive vacuole. The parasite degrades erythrocytic hemoglobin. It polymerizes the liberated toxic heme monomers to inert biocrystals of hemozoin. Chloroquine is a weak base with pKa values of 8.1 and 10.2. Therefore, a proportion of the drug remains uncharged at the neutral pH of the blood. This allows chloroquine to diffuse freely across parasite membranes. However, in an acidic digestive vacuole, chloroquine becomes deprotonated and unable to pass across the parasite membrane. As it accumulates in the digestive vacuole, it binds to hematin forming a heme dimer.

This heme dimer prevents the detoxification of free heme, leading to the accumulation of heme monomers which permeabilize the membrane. As a result, parasite death occur (Reviewed by Petersen *et al*., 2011).

Chloroquine sensitive parasites accumulate more chloroquine in the food vacuole than do resistant parasites (Fitch, 1970 cited in Saifi *et al*., 2013). This brings an assumption that chloroquine resistance parasite evolves due to insufficient access of the drug to the parasite food vacuole, but is not due to the mode of action of the drug on the parasite (Saifi, *et al*., 2013). The *Pfcrt* has different protein domains in which specific function is unique to each domain. Any mutations occurring is to these functional domains distort the functionality of the trans-membrane domain. Chloroquine resistance is associated with point mutations at this transporter gene (Keen *et al*., 2007; Jovel *et al*., 2011; Fançony *et al*., 2012). Some trans-membrane domains may have similar function. *Plasmodium falciparum* digestive vacuole membrane domain 2 and domain 1 do have similar function that is recognition and discrimination of substrate. *Plasmodium falciparum* chloroquine resistance transporter gene *(Pfcrt*, K76T) mutation occurs at domain 1 and the regular membrane function of domain 1 fails. As a result mutation which is found at a given domain will be related to the other membrane domain functions. Point mutation at codon 76 of the *Pfcrt* gene (K76T) is the substitution of threonine amino acid for lysine at position 76. This is a key molecular marker of *P. falciparum* chloroquine resistance (Mula *et al*, 2011; Atroosh *et al*., 2012). Detection of K76T mutation in the *Pfcrt*gene provides information on the chloroquine resistance status of parasites (Sharma, 2012). The (*Pfcrt*, 76T) mutation is very useful molecular marker of chloroquine resistance *P.*

falciparum, where resistance rates are low to mild (DJimde *et al*., 2001). In other words, the (*Pfcrt ,K76T)* point mutation is the first mutation that occurs in chloroquine resistant *P. falciparum* than the rest point mutation occurs at *Pfcrt* gene hence it indicates earlier mutations. When the resistance level is high other point mutations could also use as a marker for chloroquine resistant *P. falciparum*.

The (*Pfcrt*, 76T) mutation possessed the ACA (threonine) codon, indicating the presence of the (*Pfcrt*, 76T), single amino acid substitution. Chloroquine resistance level by single amino acid substitution can be enhanced by other related mutations. In other words, (*Pfcrt*, K76T) resistance level is higher when it exists with other point mutations in the chloroquine transporter trans-membrane (Setthaudom *et al*., 2011). Therefore, any mutation on the parasite membrane affects permeability of the membrane to chloroquine thereby favouring chloroquine resistance parasite domination.This mutation is primarily implicated in chloroquine resistance.

Certain mutations in its proteins and enzymes help parasite to survive drug pressure during treatment. The survived parasite populations with these selective mutations proliferate and become the best adapted parasite against the drug. The genes which encode these mutations serve as molecular markers to detect drug resistance strains (Sharma, 2012).The different level of drug accessibility to health centres or communities put different drug pressures to the parasite, which may have direct impact in prevalence of drug resistant and drug-sensitive haplotypes (Fançony *et al*., 2012). As a particular drug is withdrawn from treatment, parasites shift from resistance haplotypes to sensitive haplotypes. Similarly withdrawal of chloroquine re-

surged the high prevalence of chloroquine sensitive *P. falciparum* (Fançony *et al*., 2012).

2.3.2. Selection pressure

The heterogeneous access to drugs across health facilities has likely led to different drug pressures, which may have influenced the prevalence of drug resistant and sensitive haplotypes (Fançony *et al*., 2012). According to Fançony *et al* (2012) as chloroquine was withdrawn, a loss of the survival advantage of chloroquine resistance parasites occurred in the absence of drug pressure, leading to resurgence in the relative abundance of wild-type.

According to Gadalla *et al* (2010) mutations in the *P. falciparum* genome encompassing amino acids 72 to 76 of the chloroquine resistance transporter gene (*Pfcrt*) are associated with resistance to chloroquine and cmodiaquine. The different (*Pfcrt)* mutant haplotypes have a consistent geographical distribution. The chloroquine resistant haplotype CVIET is predominant in South East Asia and Africa (Keen *et al*., 2007). The wild-type CVIEK haplotype is widespread (Fançony *et al*., 2012). Polymorphisms in the parasite protein Pgh-1, encoded by *Pfmdr1*, are thought to modulate resistance to drugs such as quinine, mefloquine, halofantrine, artemisinin, lumefantrine, chloroquine, and amodiaquine. Mutations in this gene have some association with geographic areas.

Gadalla *et al* (2010) stated that rapid intra-host selection of particular genotypes after the introduction of drug will cause frequent misidentification of parasite genotypes

present in the starting population. This will have a serious confounding effect on clinical trials which employ PCR-corrected estimates of treatment failure. As a result, resistant parasites below the detection threshold in the pre-treatment sample can be erroneously classified as “new” infections during follow-up, over-estimating drug efficacy.

2.3.3. Parasite clearance time

Drugs have different parasite clearance time for effective treatment of malaria parasites. A few drugs delay in parasite clearance while others do have short parasite clearance time. Chloroquine has long clearance time compared to other drugs. Delay in parasite clearance time is caused by many factors. It is related to drug resistance and treatment failure in uncomplicated malaria .The long parasite clearance time is originated from the host, parasite and drug factors contributing almost equally to delay in clearance. Subsequent failure of treatment in parasites with delayed in parasite clearance time may be an indicator for emergency of drug resistance populations (Gadalla *et al*., 2010). The carriages of these mutant parasites are associated with an increased risk of treatment failures (Sowunmi *et al*., 2010).

2.3.4. Chloroquine resistance

The patterns of mutations in general adapt with the situation of long-lasting chloroquine usage before the presence of ACT (Eshetu *et al*., 2010). Chloroquine is also the first-line treatment for *P. vivax* infection; however, the prevalence of chloroquine resistant *P. vivax* is increasing. In a highly malaria-endemic region,

sufficient evolutionary changes at the molecular level are quite extensive (Sutar *et al*., 2011).The mutant haplotypeCVIET is more common where chloroquine resistance is high (Mittra *et al*, 2006). The CVIET mutant haplotype is wide spread in Africa and SVMNT mutant haplotype in Asia (Al-Hamidhi *et al., 2013*). The mutant haplotype CVIET has high level of chloroquine resistance than do SVMNT mutant haplotype under *in vitro* test (Mittra *et al*., 2006).

2.3.5. Types of point mutations

There are two main mutations, namely *Pfcrt* and *Pfmdr1* which characterize chloroquine resistance in *P. falciparum*. The chloroquine resistance mutations at digestive food vacuole membrane have an association with each other. The *Pfcrt* (K76T) mutation occurs first and then another mutation follows. The (*Pfcrt*, K76T) point mutation yields more resistance than other *Pfcrt* point mutation (Sutar *et al.,* 2011).The association of K76T mutation with other mutations in *Pfcrt* gene gives different levels of chloroquine resistance (Chaijaroenkul *et al*., 2011). The K76T mutation is selected first follows by its association with other mutations in *Pfcrt*. Association of K76T could occur with other point mutations at *Pfcrt* gene such as C72S or M74I + N75E/D. Chaijaroenkul *et al* (2011) found novel mutations at K76A. The differences in access to anti-malarial treatment are also a possible contributor to the varying levels of (*Pfcrt*, 76T) (Bin Dajem *et al*., 2010; Frosch *et al*., 2011).

2.3.6. *Pfmdr1* copy number and codon mutation

An increase in copy number of a gene may enhance the level of drug resistance. The high copy number of a target gene found together with a codon mutation, then the parasite possess enhanced anti malaria drug resistance level. A study showed that an increased *Pfmdr1* copy number with wild type codon (N86) had mefloquinine sensitivity. On the other hand, increased *Pfmdr1* copy number with codon mutation (86Y) had higher resistance level (Price *et al*., 1999). However, other recent studies showed that higher copy number of *Pfmdr1* can cause resistance in mefloquinine, artesunate and lumefantrine while low copy number is sensitive in *in vitro* test regardless of codon mutation (Sidhu *et al*., 2006). The *Pfmdr1* polymorphism showed no relationship in *in vitro* susceptibility of these drugs (Lim *et al*., 2009). *Pfmdr1* copy number variation is more frequent event than *Pfmdr1* codon mutation (Preechapornkul *et al*., 2009).

2.3.7. *Pfcrt* and *Pfmdr1* copy number

Strong association was reported between the K76T and N86Y mutations for chloroquine resistance (Sutar *et al*., 2011, Bin Dajem *et al*., 2011). When (*Pfcrt*, K76T) and *Pfmdr1* wild type (N86) occur together the parasite shows resistance to chloroquine. On the other hand, when (*Pfcrt*, K76T) occur with *Pfmdr1* mutant type (86Y) the parasite show resistance to chloroquine, but (*Pfcrt*, K76) and (*Pfmd1*, N86) does not have resistance to chloroquine. This implies that the presence of (*Pfcr*, K76T) mutation is precondition for the *Pfmdr1* parasite to develop multi-drug resistance property against chloroquine and also *Pfmdr1* increases the level of

chloroquine resistance having synergetic effect with (*Pfcrt,* K76T). High parasite population with (*Pfcrt*, K76T) mutation favour the emergence of (*Pfmdr1*, N86Y) mutation (Mittra *et al*, 2006). Chloroquine resistance reached at fixation stage after the development of resistance (Bin Dajem *et al*., 2012; Khattak *et al*., 2013).The fixed population of chloroquine resistant *P. falciparum* will be continued in areas where *P. falciparum* and *P. vivax* are co-endemic (Mungthin *et al*., 2014). In the study area both *P. falciparum* and *P. vivax* are endemic, and *P. vivax* is treated by chloroquine as first line drug while *P.falciparum* is treated by coartem. The diagnosis facility was poor so that these species could not be differentiated accurately as mixed infections or single infections. Therefore, the probability of mistreatment is high. *Plasmodium vivax* would have been treated by coartemor *P.falciparum* would have been treated with chloroquine. As a result, the resistant *P. falciparum* population is fixed and will be continued unless a change is made for effective diagnosis and treatment. A study done in China (Yunnan) showed high frequency of (*Pfcrt*, K76T) after thirty years withdrawal of chloroquine treatment for *P. falciparum*, but under continuous use of chloroquine as first line drug for *P. vivax* (Huang *et al.*, 2012).

If a particular resistance is fixed in a population for a drug chloroquine and a second drug sulfadoxine is introduced, then resistance to the second drug will occur in the parasite population on a background of prior resistance. Such multidrug-resistant strains in the population will remain stable and increase in the population as inbreeding renders chromosomal re-assortment ineffective (Griffing *et al*., 2010).The generation of these multidrug population risk the introduction of third and fourth drugs. In some cases, when the first drug is withdrawn from population and second

drug introduced, the resistance population revert to wild type and become sensitive again for the previous drug. The CVIET haplotype, which is common in Africa, is more likely to revert to chloroquine sensitivity than other SVMNT haplotypes (Griffing *et al*., 2010).

2.3.8. Chloroquine withdrawal

The prevalence of mutant type (*Pfcrt*, 76T) in Malawi, Kenya and Tanzania was high before chloroquine withdrawal, but recently its prevalence is low or disappeared. The decline in chloroquine resistance mutant type (*Pfcrt*, 76T) prevalence occurs due to the withdrawal or apparent use of chloroquine. Chloroquine resistance had disappeared in Malawi since 2003. Tanzania had a significant downward prevalence of mutant type (*Pfcrt*, 76T) and is declining in Kenya as well (Mwai *et al*., 2009; Frosch *et al*., 2011). There were no comprehensive studies done in Ethiopia on the prevalence of chloroquine resistance point mutations so far except one study done in Southern Ethiopia on prevalence of (*Pfcrt, K76T)* and (*Pfmdr1*, N86Y) (Schunk *et al*., 2006). The study showed high prevalence of both types of point mutations in Southern Ethiopia.

Malaria parasite populations dynamically evolve along with anti-malarial drug pressure. The change of treatment pressure (drug) affects the parasite evolutionary process. The drugs of malaria can be reused either as monotherapy or combination therapy after a long period of withdraw. The use of artemisinin combination therapy (ACT) increased throughout Africa (Fançony *et al*., 2012). Therefore, the drug pressure has been changed from chloroquine to artemisinin combination drugs. The

withdrawal of chloroquine could be reverted resistant *P. falciparum* to sensitive *P. falciparum* population. As a result, chloroquine with appropriate partner drugs may be used in the future possibly being limited to targeted plasmodium species (Gadalla *et al*., 2010; Frosch *et al*., 2011). The drug chloroquine could be used to treat malaria in places where the prevalence of point mutation is very low or disappeared due to abandoned chloroquine use, but in Ethiopia chloroquine is incompletely withdrawn, therefore, it may be disadvantageous to reuse chloroquine for treatment of *P. falciparum* either as monotherapy or combination therapy because previous mutations are fixed.

2.4. Microscopy and PCR methods as diagnostic tools

2.4.1. Microscopy

Microscopy is a "gold standard" method which remains the most appropriate method for malaria diagnosis in resource-limited area. Most microscopists who see one species might not look for another and misclassification is due to lack of microscopic skills to differentiate morphological variation within and between species (Zakeri *et al*., 2010; Obare *et al*., 2013). Misclassification is commonly occurring when non *P. falciparum* species are involved (Mohapatra *et al*., 2012). It is known that microscopy does not reliably distinguish the co-existence of different species (Barber *et al*., 2013).

2.4.2. Polymerase chain reaction

Polymerase chain reactor (PCR) is better tool for measuring *Plasmodium* prevalence and detection of both asymptomatic and symptomatic *Plasmodium* parasites than

microscopy. Submicroscopic or low level parasites may not be detected by the conventional microscopy (Golassa *et al*., 2013). Molecular diagnosis by real-time PCR offers a more reliable means to detect malaria parasites, particularly at low parasitaemia level and during mixed infections (Rantala *et al*., 2010;Cnops *et al*., 2010). The purpose of the current study was to re-evaluate the level of single and mixed infections of *P. falciparum* and *P. vivax* using real-time PCR.

2.4.3. Conventional PCR and real-time PCR

Real-time PCR is an advanced PCR type over conventional PCR. It is assisted by computerized software for monitoring the progress of DNA amplification. It does not depend on end product analysis unlike conventional PCR. The progress of DNA amplification can be easily observed during amplification reactions. In addition, real-time PCR is helpful for excluding primer-dimers and non-specific amplifications from target DNA amplification products. Melting curve analysis is applied in real-time PCR for PCR product identification based on; temperature profile, melting curve shapes and peaks. Amplification products can be distinguished based on colour differentiation system, particularly when multi-colour system is used in real-time PCR. It also helps to quantify target gene. Real-time PCR takes less time than conventional PCR.

CHAPTER THREE

METHOD AND MATERIALS

3.1. Description of the study area

The study was conducted in six health centres in Dembia district and one hospital in Genda-wuha district. Dembia district is located at an altitude range of 1750 to 2100m above sea level ; latitude and longitude of $12^0$36'N$37^0$28'E and 729Km from the capital Addis Ababa (Figure 3.1). Dembia district covers an area of 1270 km^2 with a total population of about 263000. This area is endemic to malaria and *P. falciparum* and *P. vivax* are the most commonly reported species from microscope diagnosis of febrile patient. The hospital is located in Genda-wuha district which is found in latitude and longitude of $12^0$58'N$36^0$12'E with an elevation of 685 meter above sea level and approximately 950km from the capital Addis Ababa.The area has approximate population of 5502. The two areas have severe malaria transmission seasons usually October to December.

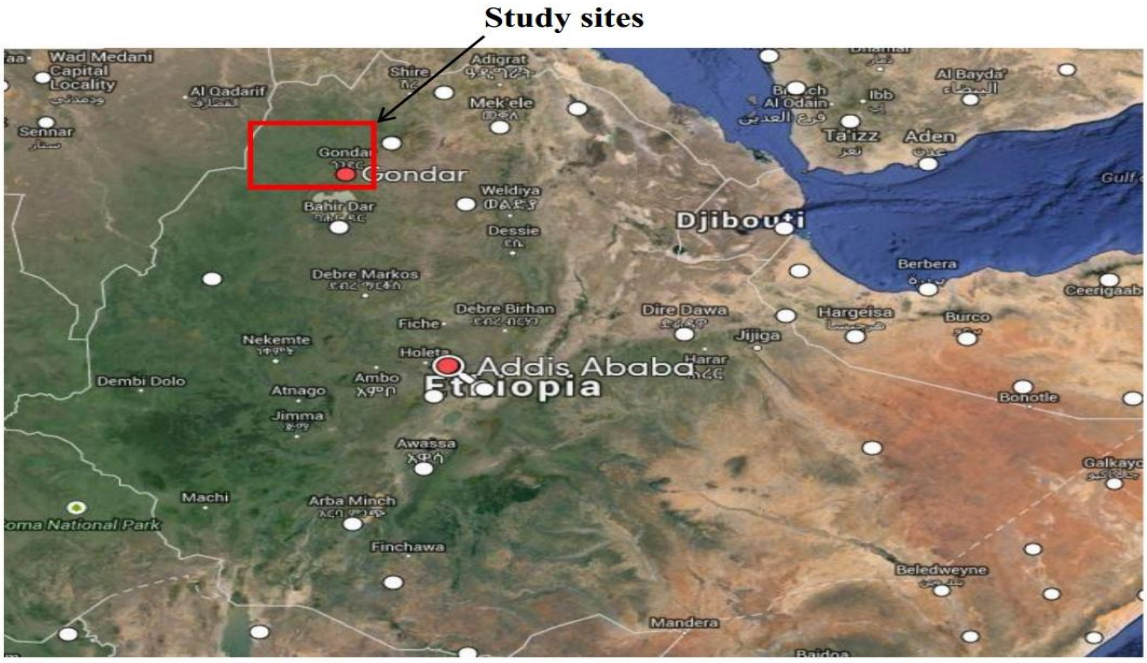

Figure 3.1Map of Ethiopia showing the study sites.

3.2. Ethical clearance

The research proposal was submitted to University of Gondar Natural and Computational Science College Ethical Review Committee for ethics approval. The Committee approved the proposed research after thorough consideration of the research proposal. Consent form was prepared for study participants. The purpose of this study was explained for participants and parents /guardians (for underage participants) in their mother tongue before being recorded in the study. Those who did not consent were not included in the study. In agreement with the clinics and the hospital the cost of drug for treatment of positive patients were covered by the clinics and the hospital.

3.3. Study design

The study was cross sectional. It was conducted during malaria transmission season (September to December, 2013). Malaria transmission is seasonal in Ethiopia, which is peak during autumn, September to December, and spring seasons, April to May. Six clinics (Tseda, Makisegnit, Chuahit, Kola Diba, Aymba and Sankisa) and one hospital (Metemma, Hospital) were used as the study sites to collect samples from outpatients.

3.4. Study participants

Patients who attended the health institutions during study period were recruited for the study. Participants, who were between six months and seventy years old, were included in the study. Infants, under six months of age and pregnant women were excluded. Patients, who were reported as *P.falciparum*and *P. vivax* positive during health service, were asked for consent to participate in the study by health professionals. Finger pricks were done, by health professionals, for those patients who consented to participate in the study.

3.5. Secondary data from microscopy

In addition to blood samples collected on filter paper, secondary data was collected from each study site. The data was collected for the second quarter malaria report of the Ethiopian fiscal year (2013) using the report format used by each health centres. This data includes *P. falciparum* and *P. vivax* single infection as well as mixed infection of both species as it was diagnosed by microscopy. During this data

collection; the total number of blood film made for malaria microscopic diagnosis, *P. falciparum* single infection,*P. vivax* single infection and mixed infections of both species were recorded from all the six institutions. Unlike the six health centres, total *P. falciparum* single infection records were obtained from the hospital. There was no any exclusion factor or criteria for the hospital in which only *P. falciparum* infection was considered, but the report was provided with only *P. falciparum* single infection. This data was collected in order to determine the prevalence level of *P. falciparum* and *P. vivax* infection in the six health centres as it was diagnosed by microscopy. Due to its severity, *P. falciparum* single infection prevalence level was considered alone in all the study sites.

3.6. Blood sampling

After finger pricking, two spots of blood samples were prepared on Whatman 3MM filter paper from each study participant. The blood spots were prepared by dropping three to four blood drops per spot on a piece of filter paper. In total, 168 blood samples were collected from all study sites (Tseda, n=14; Makisegnit, n=14; Sankisa, n=33, Chuahit, n=6; Kola Diba, n=32, Aymba, n=31; Metemma Hospital, n=38). The spots were properly labelled, air dried at room temperature. Silica gel was added inside each plastic bag to prevent humidity. In order to avoid cross contamination one plastic bag was used for one filter paper. The cards were collected in sealed plastic bag and transported to Kenya Medical Research Institute for molecular analysis. Then the samples were stored at -20^0C freezer until DNA was extracted.

3.7. Parasite DNA extraction

Apiece of dried blood spot of approximately 2 mm-3 mm in size was cut with sterile scissors. The pieces were placed in sterile 1.5 ml extraction tubes using flamed forceps. High pure PCR template preparation kit (version 20; Roche diagnostics, GmbH, Germany) was used to extract parasite DNA from dried blood spots. The extraction was done according to the manufacturer's instructions. The extracted DNA was stored in -20^{0}C freezer until used for PCR.

3.7.1. Real-time PCR condition and amplification

Real-time PCR is an advanced PCR type over conventional PCR. It is assisted by software for monitoring of the progress of DNA amplification. It does not depend on end product analysis. The progress of DNA amplification can be easily observed during the amplification reaction. In addition, real-time PCR is helpful for exclusion of primer-dimers, non-specific amplifications and, melting curve analysis is applied in real- time PCR. Real- time PCR helps to quantify the target gene and it takes less time than conventional PCR. Therefore, in order to achieve the three objectives real- time PCR was used in the study.

One step PCR was done using AccuPower 2x GreenStar^{TM} qPCR Master mix in Exicycler 96 ^{TM} (Bioneer South Korea). Exicycler^{TM} version 3.0 Software was used for programming the Exicycler Thermal Block (Exicycler 96) and data analysis was made based on operating manual from Bioneer. The reaction condition was done according to manufacturers' instruction from the AccuPower 2x GreenStar^{TM} qPCR

Master mix. It was done as first initiation step at 95^0C for 10 minutes; denaturation step at 95^0C for 20 seconds; combined annealing and extension at 55^0C for 30 seconds followed by scanning of the amplification product. This one step PCR was run for continuous 40 cycles. The PCR products were immediately subjected for melting to generate melting curves.

The melting process occurs when double stranded DNA formed after PCR amplification is dissociated at high temperature and as it melts the SYBR Green I dye fluorescent gradually decreases because the dye no longer bound to the double stranded DNA. This phenomenon detected by the Exicycler software and the software gave melting curves which had sharp turning point at the maximum melting temperature of each double stranded PCR amplification products. Therefore, each PCR product has specific "melting point" and hence temperature profiling is used for identification of PCR products.

3.7.1.1. Melting curve analysis

Melting curve analysis performed with real-time PCR detection technologies in which the fluorophore remains associated with the amplicon. An amplification that uses SYBR Green I dye is subjected to melting curve analysis.The level of fluorescence of SYBR Green I dye significantly increases upon binding to dsDNA. By monitoring the dsDNA as it melts, a decrease in fluorescence seen as soon as the DNA becomes single-stranded and the dye dissociates from the DNA.

3.7.1.2. Importance of melting curve analysis

The specificity of a real-time PCR assay was determined by using well-designed primers and reaction conditions. However, there is always the possibility that even well-designed primers form primer-dimers or amplify a non-specific product. There is also the possibility when performing quantitative real-time PCR that the sample contains nucleic acid contaminant, which would have been amplified. The specificity of the real-time PCR reaction was confirmed using melting curve analysis.

3.7.1.3. Performing melting curve analysis

Melting curve analysis was performed by programming the real-time PCR instrument to include a melting profile immediately following the thermal cycling protocol. After amplification was complete, the instrument was re-heated and the amplified products had given complete melting curve data. The melting curve peaks formed as 50% of double stranded DNA undergone melting and single or double sharp peaks formed depending on the type of template used.

3.8. Real-time PCR for *Plasmodia* species detection

The amplification of multi-copy 18SrRNA gene was employed using published primer pair 18SrRNA-F (5'-TAA CGA ACG AGA TCT TAA-3'), and 18SrRNA-R (5'-GTT CCT CTA AGA AGC TTT-3' (Kathy, *et al*., 2005). The amplification was done in 50µl reaction volume. 25µl master mix, 2µl (10pmol) forward primer, 2µl (10pmol) reverse primer, 16µl DEPC-distilled (PCR grade) water and 5µl DNA template were used. Non-template control, known positive *P. falciparum* laboratory

cultured strain *3D7,* and microscopically-positive *P. vivax* were used as control groups. Microscopically *P. vivax* positive samples were reconfirmed before being used as a control, with another independent PCR assay in which *P. vivax* specific published primer pair Pvr47-F (5'-CTT ATT TTCCGC GTA ACA ATG-3') and Pvr47-R (5'-CAA ATG TAG CAT AAA AAT CTA AG-3') were used for confirmation (Demas, *et al.,* 2011). After confirmation, *P. vivax* samples were included in each PCR run and used as control groups throughout the experiment for species differentiation.

Mixed infections occur in nature at different parasite load of each species per infection. Therefore, an artificial mix of *P. falciparum* and *P. vivax* DNA were made at initial concentration of 15.75ng/µl in different volume ratios. The mix was done in order to mimic natural mixed infections which occur in an imbalanced parasite load per infection. The mixing was done in the following ratios: (100µl:0µl, 75µl:25µl, 50µl:50µl, 25µl:75µl, 0µl:100µl). The mixing of the two species was made to obtain the optimum detection threshold of the assay for mixed infections. The minimum detectable concentration of the template DNA in the mixture was also determined indirectly from the non-template control "well" from the reaction plate because this well has only primers concentrations. The melting curves obtained from artificial mixed species and melting curves obtained from natural mixed infections from clinical samples were compared for confirmation purpose. Thirty-four (20%) of the samples were replicated twice to validate reproducibility of the experiment.

3.8.1. Melting curve analysis for species detection

After amplification the product was immediately subjected for melting at 70^0C to 94^0C at ramping rate of 1.0^0C for 2 seconds. In order to detect *Plasmodium* species based on respective temperature profile the primary differential (F') was selected from melting curve window. The shape and peaks of the melt curve was carefully observed. The dual and single melting curve peaks were observed from 73^0C to 82^0C.The most curve peaks were clustered from 77^0C +/- 1.The melting curve peaks were shown as discrete variable values. If the curve was observed as dual sharp peaks with more than a difference of 1.5^0C, the peaks were recorded as mixed infection. If the curve was observed as a single sharp peak the result was recorded as mono-infection. *Plasmodium falciparum* infection was represented at 75^0C to 78^0C temperature range. *Plasmodium vivax* mono-infection was represented at peaks lying on 80^0C or above. *Plasmodium ovale* was recorded at 79^0C. If dual peaks at 77^0C and 80^0C observed then it represented mixed infection of *P. falciparum* and *P. vivax (*Kathy *et al.,* 2005*).*

The real-time PCR results were recorded from amplification curves and melting curves after each run of PCR. The amplification curves in threshold and log scale format were obtained from the Exicycler software in order to observe target gene amplification and the presence of primer-dimers, non- specific amplifications, respectively. The amplification curves in log scale form clearly present the exclusion of the "noise" or non-specific amplifications and primer-dimer in the left bottom side

of the curves. The default auto-threshold and log scale form were used to obtain the amplification curves.

3.9. Real-time PCR amplification for (*Pfcrt, K76T)* identification

The amplification of 275bp fragment of (*Pfcrt*, K76T) gene was employed using published primer pairs CRT-F (5'-TGA CGA GCG TTA TAG AG-3') and CRT-R (5'-GTT CTT TTA GCA AAA ATT G-3') (Wurtz, *et al*., 2012)**.** The amplification was done at 25µl reaction volume. 12.5µl AccuPower 2x GreenStar^{TM} qPCR Master mix, 2µl (10pmol) forward primer, 2µl (10pmol) reverse primer, 3.5 µl DEPC-distilled water and 5µl DNA template were used. Non-template control was included to control formation of primer-dimers. Chloroquine resistant and sensitive *P. falciparum* strains (*W2, D6* and *3D7*) obtained from Kenya medical research institute malaria laboratory were used as controls in order to identify wild type and mutant type clinical samples. These controls were included in each PCR run throughout the experiment. An artificial mix of DNA extracted from these strains were made in different ratio in order to determine the efficiency and sensitivity of the assay to identify the wild type (*Pfcrt*, K76) and mutated type (*Pfcrt*,76T) at different concentration levels. The ratios of strain *W2* to strain *D6* were (10µl:10µl, 5µl:10µl) and strain *W2* to strain *3D7* were (10µl:10µl, 5µl:10µl). The mixing of the two strains was made to obtain the optimum detection threshold of the assay for genotyping (*Pfcrt*, K76T). The minimum detectable concentration of template DNA was indirectly determined from the Ct values of the amplification graphs by comparing it with the non-template control "well" from the reaction plate. The melting curves

obtained from artificial mixed resistant strain with sensitive strain, and melting curves obtained from clinical samples were compared for confirmation purpose.

3.9.1. Melting curve analysis for *Pfcrt (K76T)* identification

Melting curve analysis is useful for identification of wild and mutant genotypes of *Pfcrt, K76T*.Since the melting curve shapes and peaks are different for these two genotypes the curve and specific temperature profile enable to discriminate wild type (*Pfcrt*, K76) and mutant type (*Pfcrt*, 76T) genotypes. Therefore, after amplification of the template DNA the PCR product was immediately subjected for melting at 70^0C to 83^0C at ramping rate of 1.0^0C for 1 second. The known positive controls were included in order to identify the wild type and mutant type clinical samples. Chloroquine sensitive strains (*D6*, *3D7*) and resistant strain (*W2*) were used as positive controls. Non-template control was also included for each run. The samples were identified as wild type and resistant type based on respective temperature profile and the shape of the melting curve graphs. The primary differential (F') was selected from melting curve window for identification of the graph shape and peaks. The shape and peaks of the melting curves were carefully observed. The dual and single melting curve peaks were observed from 70^0C to 83^0C.

3.9.2. *Pfmdr1*(N86Y)and copy number determination by qPCR

Real time PCR quantification of the target gene is better quantified when the template is short usually less than 200bp. Therefore, in this study the *Pfmdr1* amplification and copy number variation was determined from 178bp long template DNA. In order to

obtain short template newly designed primer pairs (Forward 5'-GAT GGT AAC CTC AGT ACT-3'and Reverse 5'- CTC CTG ATA ATA CAG CAC-3') were used with dual labelled single probe (5'-TET-ACC TAA ATA CAT GTT CTT T-3'-BHQ1). The probe was used to differentiate the mutant and wild type of the samples at *(Pfmdr1, N86Y)* codon. The labelled probe complements to the codon mutation specific region of the *(Pfmdr1, N86Y).* Fluorescence can be detected from the complementation of the Probe with template because the quencher and reporter dyes fluoresce only when there is complementation between the target and the probe sequences. The probe sequence was obtained from published paper (Purfield *et al*., 2004).

In order to determine the copy number variation, targeted in (*Pfmdr1, N86Y)* position, these newly designed qPCR primer pairs flanking in the complete mRNA (CDS) regions of *P. falciparum* (*3D7* strain) at chromosome five were used. The *Pfmdr1* or Polyglycoprotein1 (*Phg1*) is coded by the gene located at chromosome five in *P. falciparum* genome and is responsible for multidrug resistance development in *P. falciparum*. The *3D7* strain sequence was selected for primer design because this strain is fully sequenced and representative for *P. falciparum* genome study. The *Pfmdr1* develop due to functional Polyglycoprotein1 (*Phg1*), therefore, non-functional sequences were excluded during the primer design.

The FASTA sequence was down loaded from NCBI (www.ncbi.nlm.nih.gov/) at GenBank accession number(XM_001351751) and pasted in the Integrated DNA Technology Primer Quest (www.idtdna.com). After copy-pasting the FASTA

sequence format the parameters such as Primer length, product length ,G+C content, Tm of primer, Tm of product and salt concentration were adjusted from drop down menu of the software. After designing the new primer pair then it was analysed for Tm mismatch, self-dimer formation (homo-dimer), hairpin formation, and hetro-dimerization. The analysis was done by IDT Oligo analyser online (www.idtdna.com) and finally, "blastn" was done for the new primer pair from NCBI. After these all process the primer pairs were validated to be used for absolute quantification of the target gene for copy number variation.

The amplification of (*Pfmdr1*,N86Y) was done at 25µl reaction volume of which 12.5 µl AccuPower 2x GreenStar^{TM} qPCR Master mix, 2 µl (10pmol) forward primer, 2 µl (10pmol) reverse primer, 2µl (10pmol) single probe, 1.5µl DEPC-distilled water and 5ul DNA template were used. Non-template control was included to control formation of primer-dimers.

Chloroquine resistant and sensitive *P. falciparum* strains (*Dd2*, *W2, D6* and *3D7*) obtained from Kenya medical research institute malaria laboratory were used as controls in order to determine copy number variation in clinical samples. These controls were included in each PCR run throughout the experiment in triplicates.

3.9.2.1. Absolute quantification of *Pfmdr1* (N86Y) copy number variation

The standard curve was built from *P. falciparum g*DNA. The supporting manual from (www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf) was obtained and used for preparation of the standard curve from strain *3D7* gDNA. *Plasmodium*

falciparum (strain *3D7*) was used as reference copy number. This strain is known to have a single copy of *Pfmdr1* gene in the haploid *P. falciparum* genome. In order to build the standard curve the concentration of DNA extracted from *3D7* was measured by Nano $Drop_{2000}$ spectrophotometer. The average concentration of the DNA was 15.75ng/µl which is equal to 15,750 pg/µl. Absorbance of the DNA sample was measured at A_{260}, A_{280}, $A_{260/280}$, $A_{260/230}$.

Derivation of DNA mass formula

$$m = \lfloor n \rfloor \left\lfloor \frac{1}{6.023e^{23}\text{Moleculules(bp)}} \right\rfloor \left\lfloor \frac{660\text{g}}{\text{mole}} \right\rfloor = \lfloor n \rfloor \left\lfloor 1.096\ e^{-21} \frac{\text{g}}{\text{bp}} \right\rfloor$$

$$m = \lfloor n \rfloor \left\lfloor 1.096\ e^{-21} \frac{\text{g}}{\text{bp}} \right\rfloor$$

Where: n = genome size (bp)

m= mass

The Avogadro's number is equal to $6.023x10^{23}$ molecules per mole and Average MW of a double-stranded DNA molecule is 660 g/mole. The size of *P. falciparum* genome is around 23Mbp (Gardner, *et al*., 2002) which is equal to 23,000,000bp. In order to obtain the mass of *P. falciparum* haploid genomic DNA from the whole genome size: 23,000,000bp was inserted for n- value in the above formula. The mass of *P. falciparum* haploid genome is calculated as follows:

$$m = \lfloor 23 \times 10^{6}\text{bp} \rfloor \left\lfloor \frac{1.096 \times 10^{-21}\text{g}}{\text{bp}} \right\rfloor = 0.025 \times 10^{-15}\text{g}$$

$$\mathrm{m} = \lfloor 0.025 \times 10^{-15}\mathrm{g} \rfloor \left\lfloor 1 \times 10^{12} \frac{\text{picogram}}{\mathrm{g}} \right\rfloor = 0.025\mathrm{pg}$$

Dividing the mass of the haploid genome by the copy number of the gene of interest per haploid genome gives the copy number of the gene in 0.025pg. The *3D7 P. falciparum* strain has the target gene that exists as a single copy gene per haploid genome (or two copies per *P. falciparum* cell). This implies that:

$$\frac{0.025\mathrm{pg}}{\text{haploid genome}} \div \frac{\text{one copy pfmdr1}}{\text{haploid gemone}} = \frac{0.025\mathrm{pg}}{\text{one copy of } Pfmdr1}$$

Therefore, 0.025 Pico gram of *P. falciparum* haploid genome contains one copy of *Pfmdr1* (N86Y) gene. Then, calculations of the mass of gDNA containing the copy numbers of interests, which are 800,000 to 0.8 copies, were done. If one copy of *Pfmdr1* gene is obtained from 0.025pg of haploid genomic DNA, then 800,000 copies of *Pfmdr1* gene can be obtained from 20,000pg.

$$\text{Copy number of interest} \times \text{mass of haploid genome} = \text{mass of gDNA needed}$$

The following calculation is used to obtain mass of haploid genomic DNA needed and the concentration of the DNA samples in five microliter template DNA in the ranges of copy numbers of interest from 800,000 to 0.8 copies. The multiplication of copy number of interest by 0.025pg gives the mass of haploid genome needed to get the concentration of DNA needed at five microliter template. The division of mass of haploid genome to five microliter DNA template gives final concentration of DNA for each dilution (table 3.1).

Table 3.1Mass of gDNA needed and final concentration of input DNA at 5µl template DNA

Copy number of interest	Haploid genome of *P.falciparum* in Pico-gram	Mass of needed (pg)	Volume of template	Final concentration (pg / µl)
800,000	X 0.025pg(haploid genome of *P. falciparum* in Pico-gram *)*	20,000	/5 µl	4000
80,000		2,000		400
8000		200		40
800		20		4
80		2		0.4
8		0.2		0.04
0.8		0.02		0.004

Ten-fold serial dilution of the stock DNA was obtained by using the following formula based on the above concentration of template DNA.

$$C_1V_1=C_2V_2$$

Where: C_1= stock concentration (15750pg/µl)

V_1= required volume

V_2=100µl

C_2=4000pg/µl

$$V1 = \frac{C2V2}{C1}$$

Then the value of V_1 = 25.4µl. In order to obtain the 100µl dilution a difference of 25.4µl from 100µl diluent is needed. The volume of diluent is therefore 74.6µl. Ten-fold serial dilution was prepared from the stock DNA (table 3.2) and standard curve was built from the diluted DNA sample (figure 4.16).

Table 3.2Serial dilutions from DNA stock solution

S.N	Initial Concentration (pg/ µl)	Volume 1(µl)	Final Concentration (pg/µl)	Volume 2(µl)
Stock	15750	25.4	4000	100
Dil.1	4000	10	400	100
Dil. 2	400	10	40	100
Dil. 3	40	10	4	100
Dil.4	4	10	0.4	100
Dil.5	0.4	10	0.04	100
Dil. 6	0.04	10	0.004	100
Dil. 7	0.004	10	0.0004	100

The standard was included in each run. The average amplification efficiency was 106% including at least three points from the serially diluted standard DNA template on the linear regression line. The PCR efficiency value is acceptable in the range

100+/-10%.Outlier points were omitted from the reaction plate in order to get appropriate efficiency. The samples values were laid on the linear regression line and there was no any outlier points omitted from the values. There was no PCR efficiency difference between the standard and clinical samples. Because, the primer pairs were the same and designed for the same target (*Pfmdr1, N86Y*) region in the calibrator strain *3D7* and *P. falciparum* positive samples. Input DNA concentration was determined automatically for each sample by the Exicycler software based on the standard concentration. The following formula was used in order to calculate the copy number of samples from the concentration of the input DNA per microliter (www.scienceprimer.com).

$$\text{DNA copy number} = \frac{\lfloor 6.023 \times 10^{23}\text{copy/mol} \rfloor \text{x} \lfloor \text{DNA amount(g/µl)} \rfloor}{\lfloor \text{amplicon length(bp)} \rfloor \text{x} \lfloor 660\text{g/mol} \times 1/\text{bp} \rfloor}$$

$$\text{DNA copy number} = \left\lfloor \frac{6.023 \times 10^{23}\text{copy/mol}}{117480\ g/mol} \right\rfloor \times \lfloor \text{DNA amount(g/µl)} \rfloor$$

The copy numbers were calculated using the above formula from DNA input concentration which was determined by the PCR; Molecular weight of DNA is 660gram per mole per base pair and the amplicon length is 178 in base pair. Finally, the copy number ratio of positive controls (*Dd2, W2* and *D6*) to *3D7* and samples to *3D7* were calculated manually in order to obtain copy number variation or fold difference between the calibrator *3D7* strain, positive controls and clinical samples. In other words the ratio of input template concentration of the samples and positive controls to the input template concentration of the *3D7* strain was determined. The

ratio gave the fold difference between the sample and the reference strain *3D7*. The *3D7* strain has single copy of the *Pfmdr1* gene per haploid genome. Therefore, if the samples had more copy number values of the target gene *Pfmdr*1 (N86Y) than the reference strain *3D7,* and then the samples were considered as multi-copy variants. In contrast, if the samples had less or equal copy number values of the target gene with the reference strain *3D7,* then the samples were considered as single copy number variants. For instance, the copy number of *3D7* strain at 4000pg/µl input concentration of DNA template was calculated as follows:

$$3D7 \text{ strain DNA copy number} = \frac{\left\lfloor 6.023 \times \frac{10^{23}\text{copy}}{\text{mol}} \right\rfloor \text{x} \left\lfloor 4000 \left(\frac{\text{pg}}{\text{ul}}\right) \right\rfloor}{\lfloor 178(\text{bp}) \rfloor \text{x} \left\lfloor \frac{660\text{g}}{\text{mol}} \times \frac{1}{\text{bp}} \right\rfloor}$$

$$= 2.05 \times 10^{10} \text{ copies } /\mu\text{l}$$

$$\text{The ratio } 3D7 \text{ to } 3D7 \gg \frac{3D7}{3D7} = \frac{2.05 \times 10^{10}}{2.05 \times 10^{10}} = 1 \text{ copy}/\mu\text{l}$$

The copy number of the positive control *Dd2* strain at 694pg/µl input concentration of DNA template was also calculated. Then, the ratio of *Dd2* to *3D7* was obtained by dividing the copy number of *Dd2* to copy number of *3D7* strain in order to get the fold difference between the two *P. falciparum* strains.

$$Dd2 \text{ strain DNA copy number} = \frac{\left\lfloor 6.023 \times \frac{10^{23}\text{copy}}{\text{mol}} \right\rfloor \text{x} \left\lfloor 694 \left(\frac{\text{pg}}{\mu\text{l}}\right) \right\rfloor}{\lfloor 178(\text{bp}) \rfloor \text{x} \left\lfloor \frac{660\text{g}}{\text{mol}} \div \frac{1}{\text{bp}} \right\rfloor}$$

$$= 3.56 \times 10^{10} \text{ copies } /\mu\text{l}$$

$$\text{The ratio } Dd2 \text{ to } 3D7 = \frac{\text{Dd2}}{\text{3D7}} = \frac{3.56 \times 10^{10}}{2.05 \times 10^{10}} = 1.736 \text{ fold}$$

The remaining values for clinical samples are computed in the same way in order to get the copy number variations.

3.10. Statistics

Statistical analysis was used for analysing the data. The prevalence level of mixed infection and misdiagnosed plasmodium species were analysed by using percentage statistics. Chi-square statistics was used in order to analysis the significance of *Pfcrt* (K76T) and *Pfmdr1* (N86Y) copy number variations prevalence.

CHAPTER FOUR

RESULTS

4.1. Microscopic results

Out of the 7,343 febrile patients who were attending the outpatient department for malaria treatment, 1,802 were reported to have either *P. falciparum* or *P. vivax* single infection or a mixed infection of both species. The report did not include *P. ovale* and *P. malariae* infections because these two species were not detected by microscopy during the study period. Among these positive cases 1,216 (67.48%) were infected with *P. falciparum*, 553 (30.68%) with *P. vivax* and 33 (1.83%) with both species (figure 4.1).

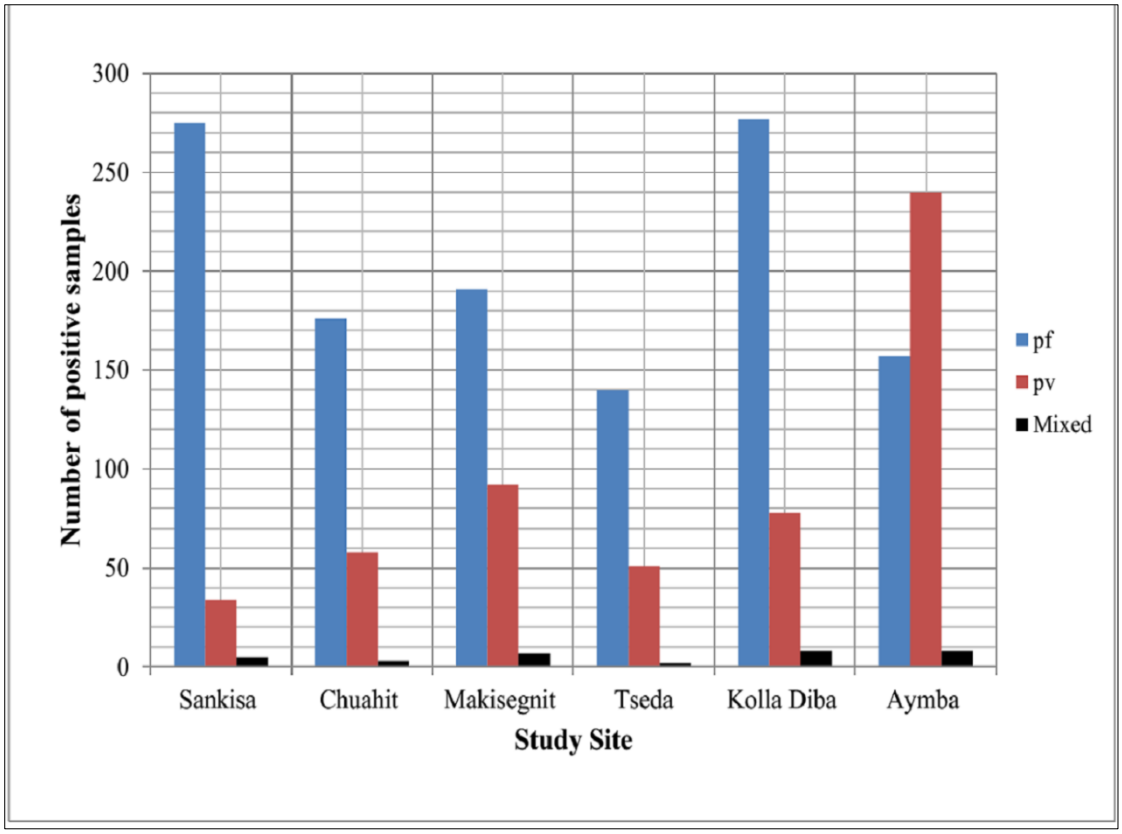

Figure 4.1 The prevalence of (*Pf*) and (*Pv*) by microscopy in the six study sites

Among the 1,802 positive patients 0.83% were below one years old infants, 6.71% were children between one and four years of age, 12.93% were between five to fourteen years of age. The remaining 79.52% were above fifteen years of age. This result showed that an increment of the prevalence of the two species as the age increased from one to fifteen and above years old patients, which suggests that the different exposure rate of infections with regard to age group (figure 4.2).

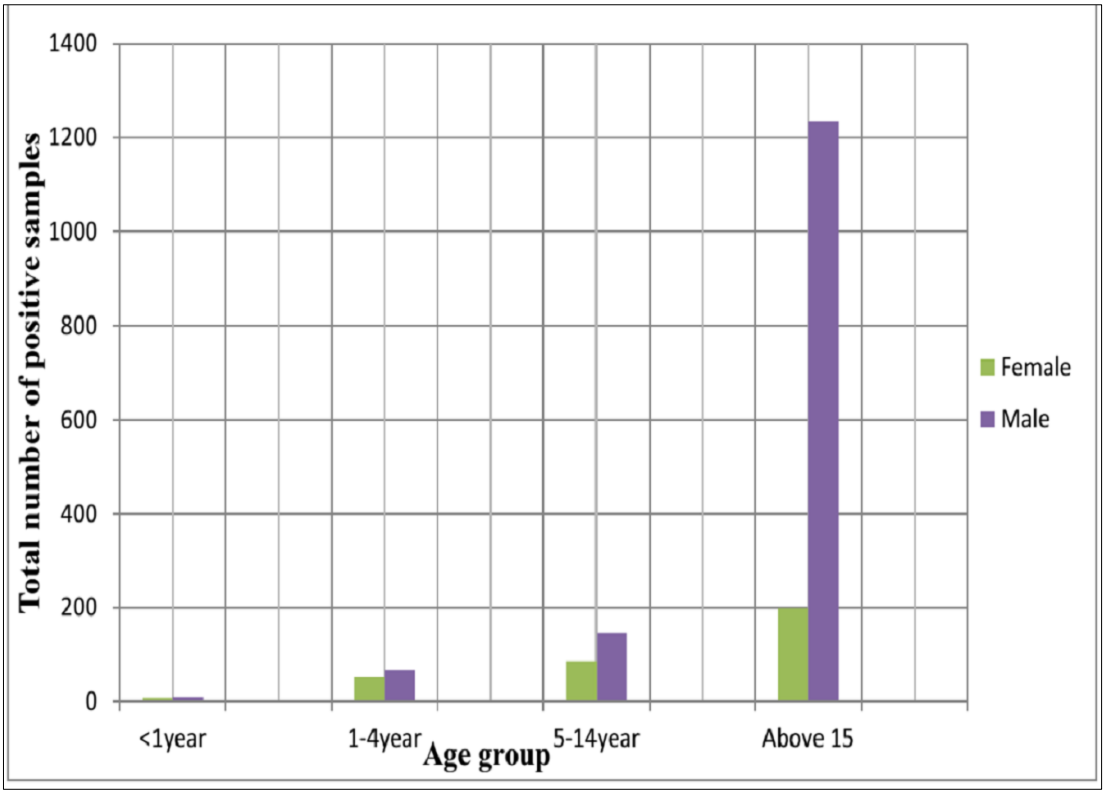

Figure 4. 2Prevalence of (*Pf)* and (*Pv)* by microscopy with regard to sex and age group

Among the 1,802 positive patients 80.85% were males and 19.15% were females. Males are field workers in the study area and more infected than females which indicated that males were more frequently exposed to mosquito bite.

The prevalence of *P. falciparum* single infection was considered in all the study sites. The maximum prevalence of *P. falciparum* was recorded in Metemma Hospital (Shedi) and the minimum was recorded in Tseda clinic (figure 4.3).

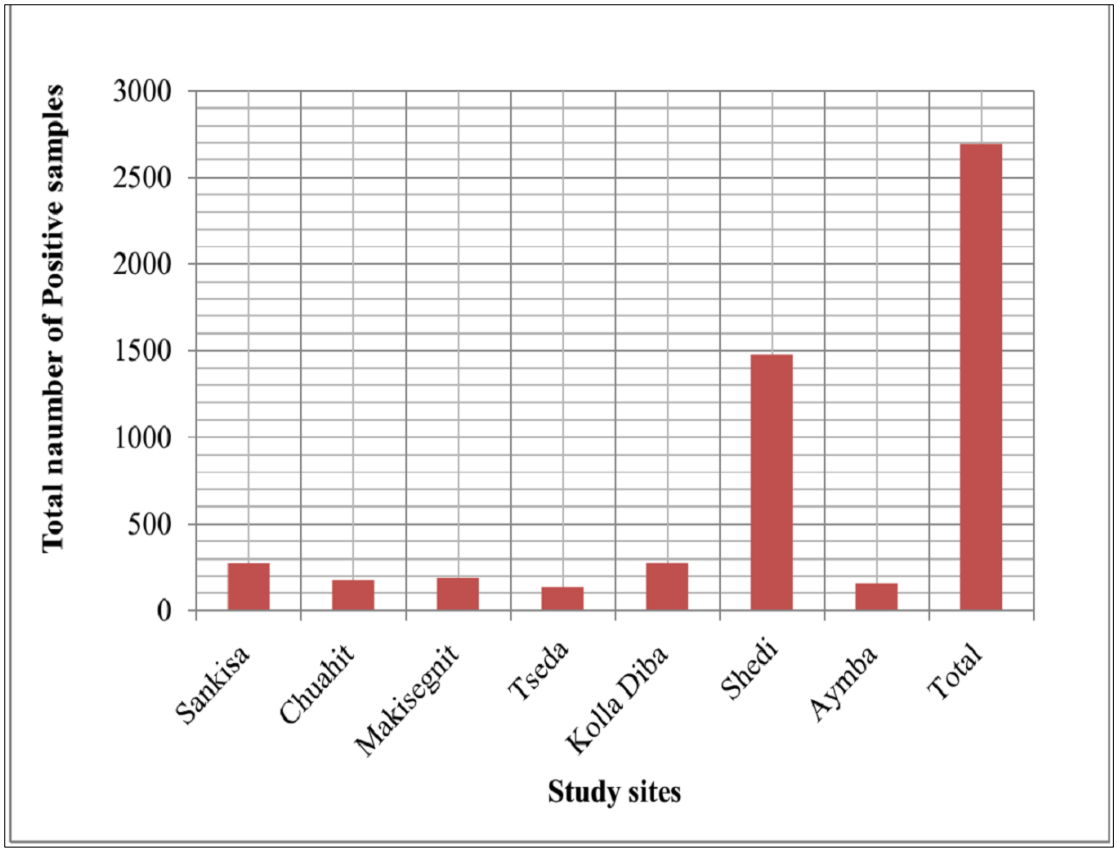

Figure 4. 3 The prevalence of *(Pf)* single infections by microscopy in all the study sites

4.2. Optimization for PCR conditions

Polymerase chain reaction optimization steps were done before the real experimental data was obtained from the amplification of all the target genes by the real-time PCR. These optimization steps were used to confirm the accurate complementation between primers and their target genes such as 18S rRNA gene for species detection, *Pfmdr1* gene for copy number determination and *Pfcrt* gene for point mutation detection. Some of the samples were used in conventional PCR and gel-electrophoresis was done for those samples for confirmation purpose. In order to check the presence of

real-time PCR product a few post real- time PCR samples were run by gel-electrophoresis, including the non-template control (N), 1000bp ladder (M), a known *P. falciparum* strain (positive control, P).

Plasmodium species detection was done by conventional PCR before the use of real-time PCR for confirmation of the complementarity between 18S rRNA gene and its primer. Microscopically *P. falciparum* positive samples (L1 to L11), non-template control (N), 1000bp ladder (M) and a known laboratory culture *P. falciparum* strain (positive control, P) were run on a gel (figure 4.4).

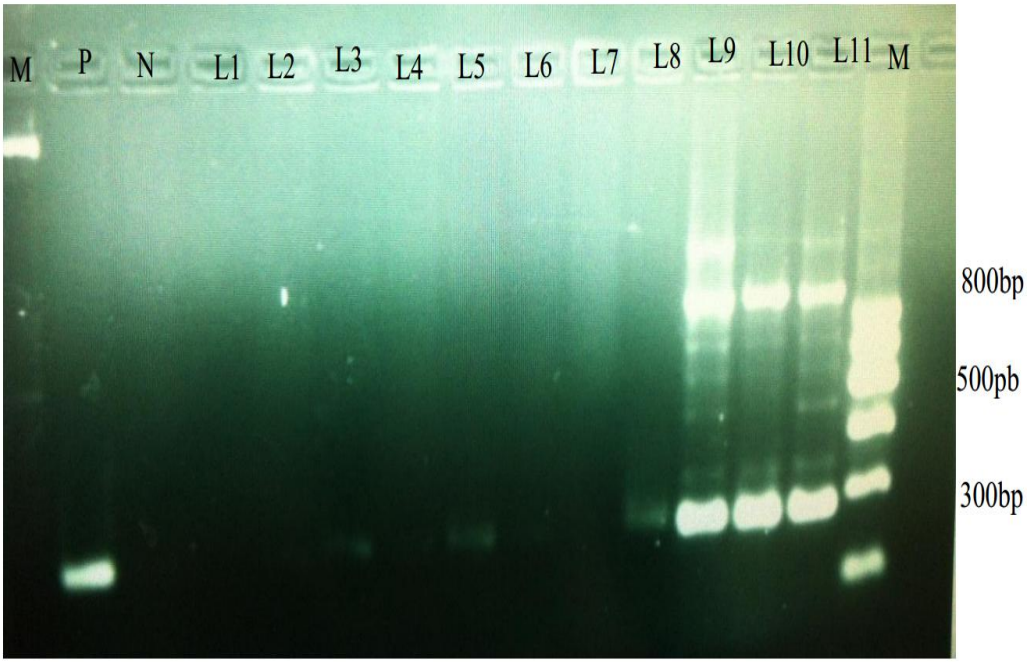

Figure 4. 4Microscopically (*Pf)* positive samples (L1 to L11) was amplified by conventional PCR and run on a gel

The wild type and mutant type (*Pfcrt, K76T)* amplification was done by conventional PCR for confirmation of complementarity between target *Pfcrt* gene and its primers.

The PCR product was run on a gel with non-template control (N), 1000bp ladder (M) and a known chloroquine resistant *P. falciparum* strain (positive control, P). The following results were obtained from the amplification of *Pfcrt* gene (figure 4.5).

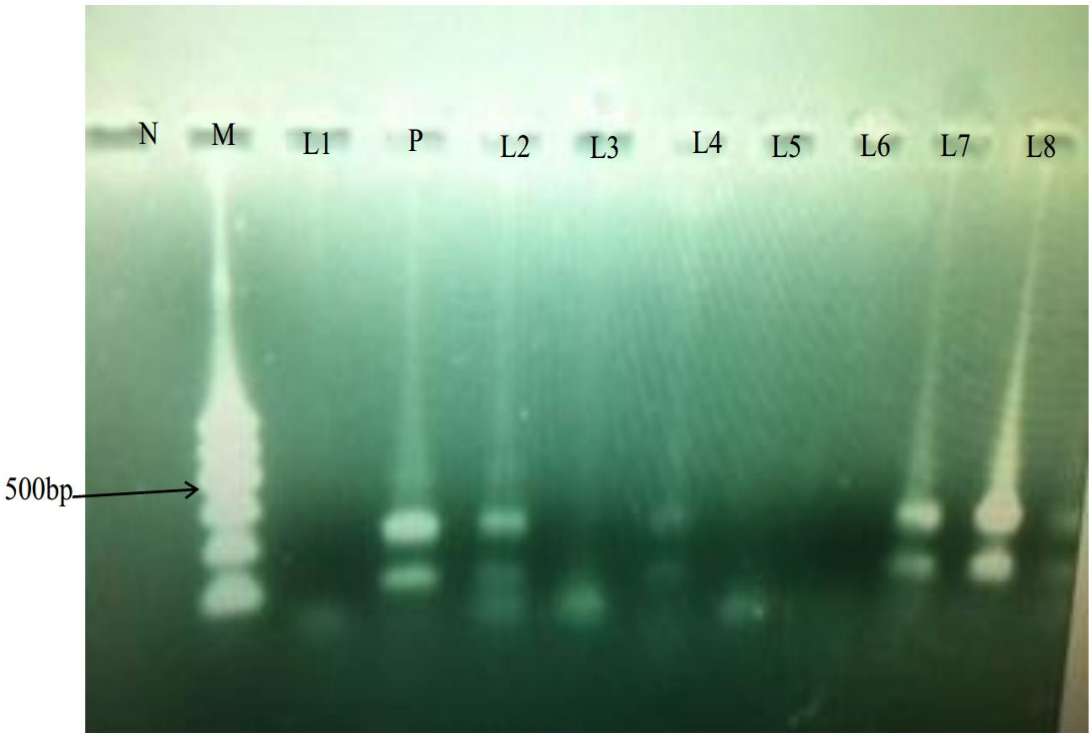

Figure 4. 5*Pfcrt (K76T)* gene fragment amplified by conventional PCR and run on a gel (L1 to L8)

Real-time PCR optimization was also performed for *Plasmodium* species detection. The template DNA obtained from microscopically *P. falciparum* positive samples were amplified by real-time PCR. The amplification products obtained from the real-time PCR was run on gel-electrophoresis for confirmation of complementarity between the target gene (18S rRNA) and its primers. A 1000bp ladder (M), non-template control (N), a known chloroquine resistant *P. falciparum* strain (positive

control, P) were included during the run for assuring the gel bands are obtained from the template DNA (figure 4.6).

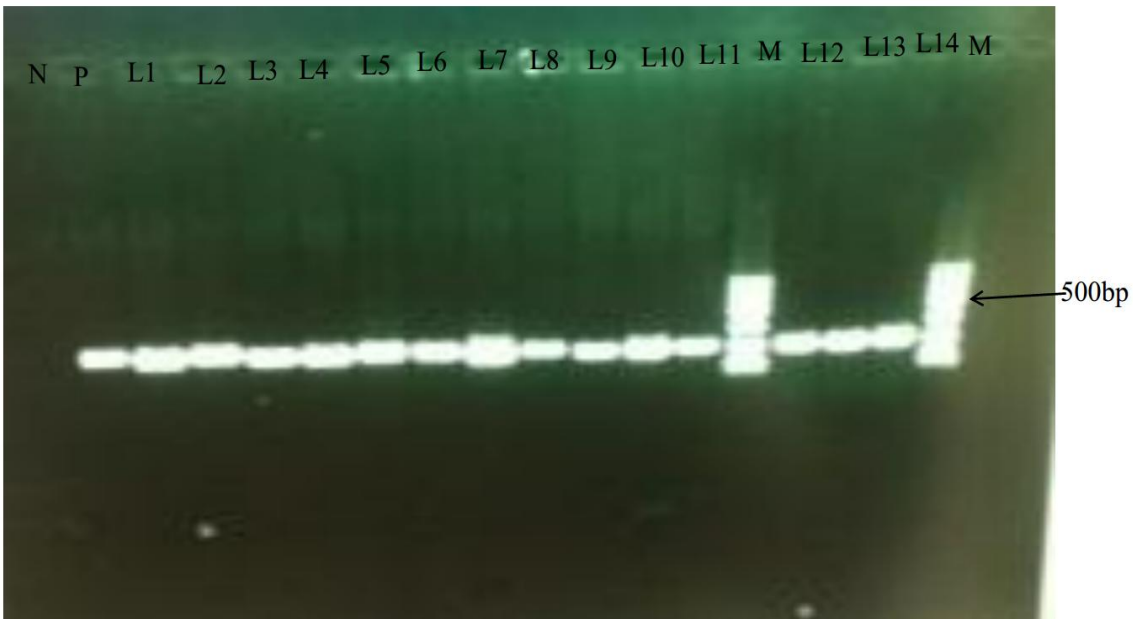

Figure 4. 6Microscopically *(Pf)* positivesamplesamplified by real-time PCR (L1 to L14) and confirmed by gel

Real-time PCR optimization was performed for (*Pfcrt, K76T*) genotyping. The *Pfcrt* gene was amplified by real-time PCR. The amplification products obtained from the real-time PCR was run on gel-electrophoresis for confirmation of complementarity between the target gene and its primers. The non-template control (N), 1000bp ladder (M) and a known chloroquine resistant *P. falciparum* strain (positive control, P) were included during the run for assuring the gel bands are obtained from the template DNA (figure 4.7).

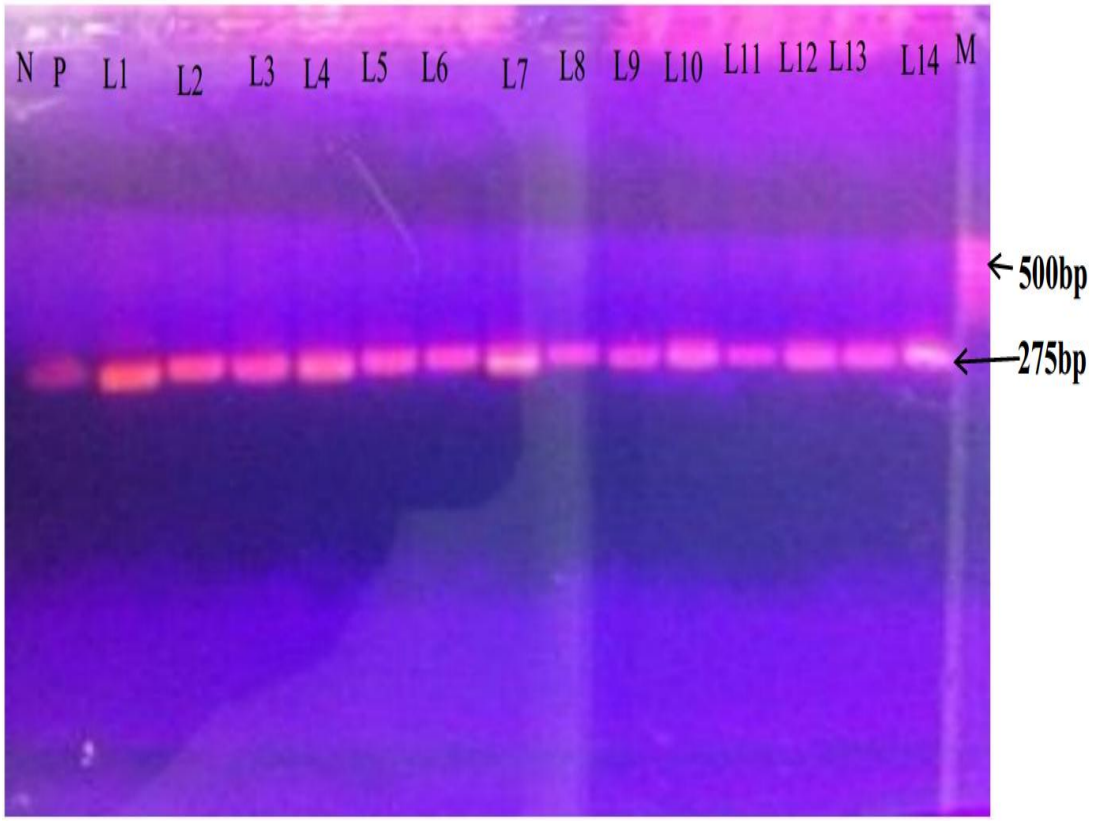

Figure 4. 7*Pfcrt* (K76T)gene fragment amplified by real-time PCR (L1 to L14) and confirmed by gel

4.3. SYBR Green I based real-time PCR results

In order to detect *Plasmodia* species multi-copy of 18S rRNA target gene was amplified with both positive and negative controls. The amplification of each clinical samples and controls were obtained from amplification graphs in log scale form. The amplification of 18S rRNA gene was obtained in log scale format in order to exclude primer-dimers, non-specific amplification and/or unexpected artefacts. The

amplification curves in log scale form avoid the primer-dimers, non-specific amplifications separately from the target amplification curves (figure 4.8).

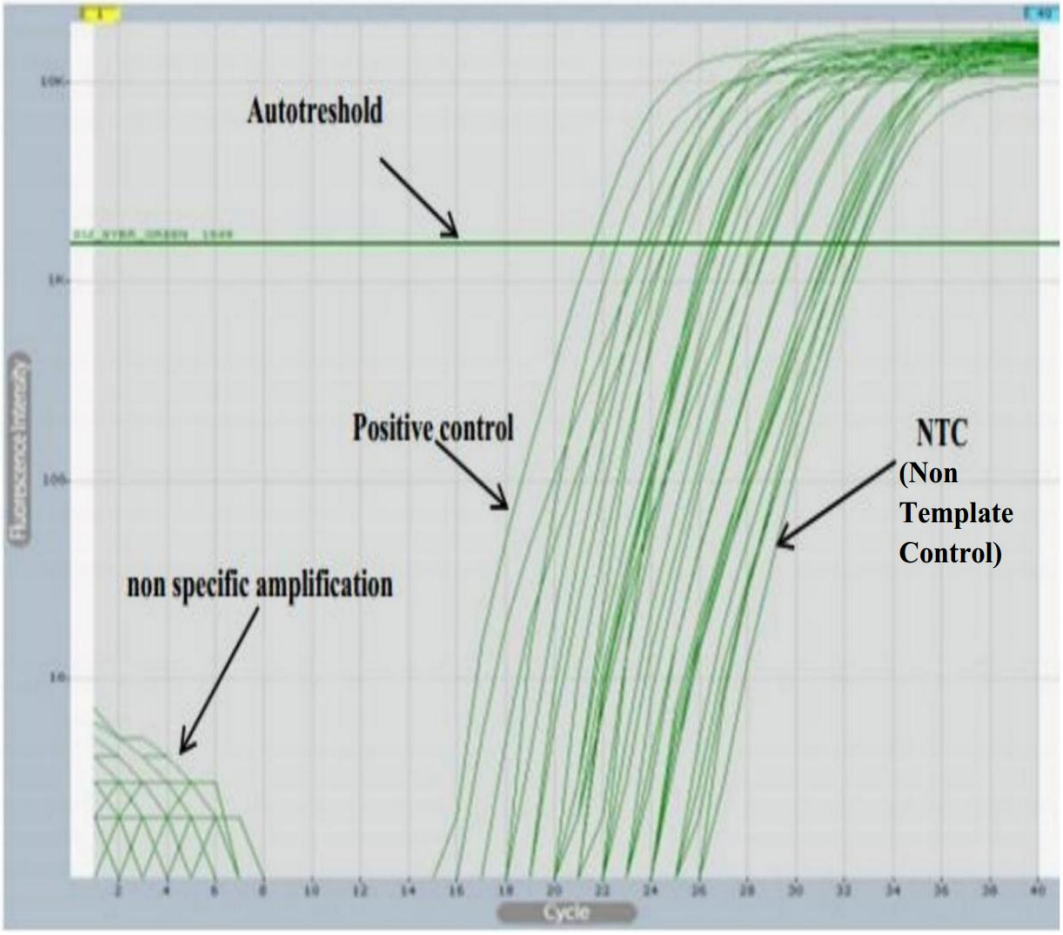

Figure 4. 8Amplification curves of 18S rRNA gene in log scale form

The melting curve analysis results obtained for each species were recorded as follows. The single peak of an amplification curve at a specific temperature represented as a single infection. In contrast, dual peaks at different temperature are represented as mixed infections. In order to validate and accurately represent each species at a

specific temperature profile on a melting curve, known positive controls such as *P. falciparum* and *P. vivax* were included. The confirmation of *Plasmodium ovale* was done from experimental set up as it was represented by previously published paper which used the same primer pair and the same melting temperature profile at 79^0C (Kathy *et al*., 2005). The confirmation of mixed infections was done by including an artificial mixed species of both *P. falciparum* and *P. vivax* at different concentrations. The temperature and dual melting curve peaks obtained from artificially mixed species were used for comparing with melting curves obtained from naturally occurring mixed infections in the clinical samples.

The single infections of *P. falciparum* and mixed infections of *P. falciparum* with *P. vivax* are represented at different temperature profiles and melting curve peaks. The single infections of *P. falciparum* are clustered around at 77^0C, and mixed infections at 76^0C and 81^0C (figure 4.9).

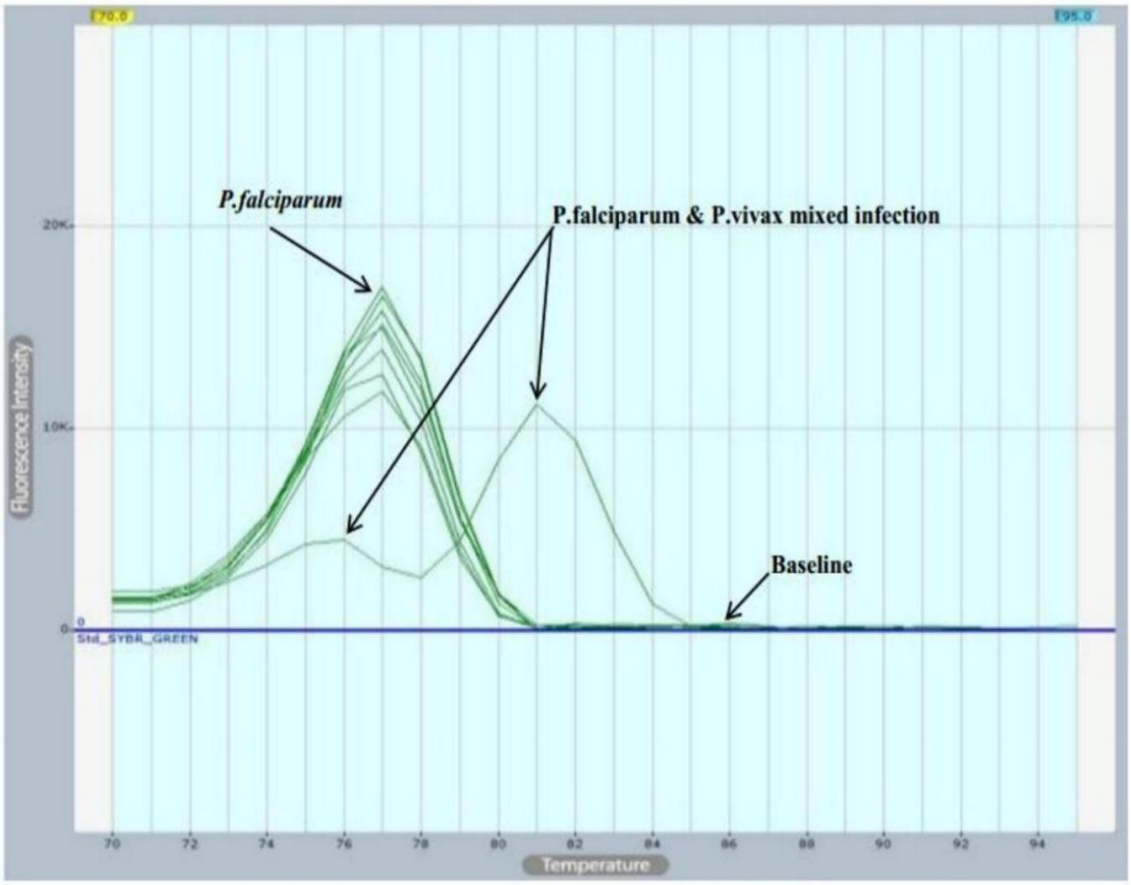

Figure 4. 9Melting curve peaks of single and mixed *plasmodium* species (*Pf*) at 77^0C and (*Pf*) + (*Pv*) at 76^0C and 81^0C respectively

The single infections of *P. vivax* and mixed infection of *P. vivax* with *P. falciparum* are represented at different temperature profiles and melting curve peaks. The single infections of *P. vivax* are clustered at around 81^0C and *P. vivax* mixed infection with *P. falciparum* is at 76^0C and 82^0C (figure 4.10).

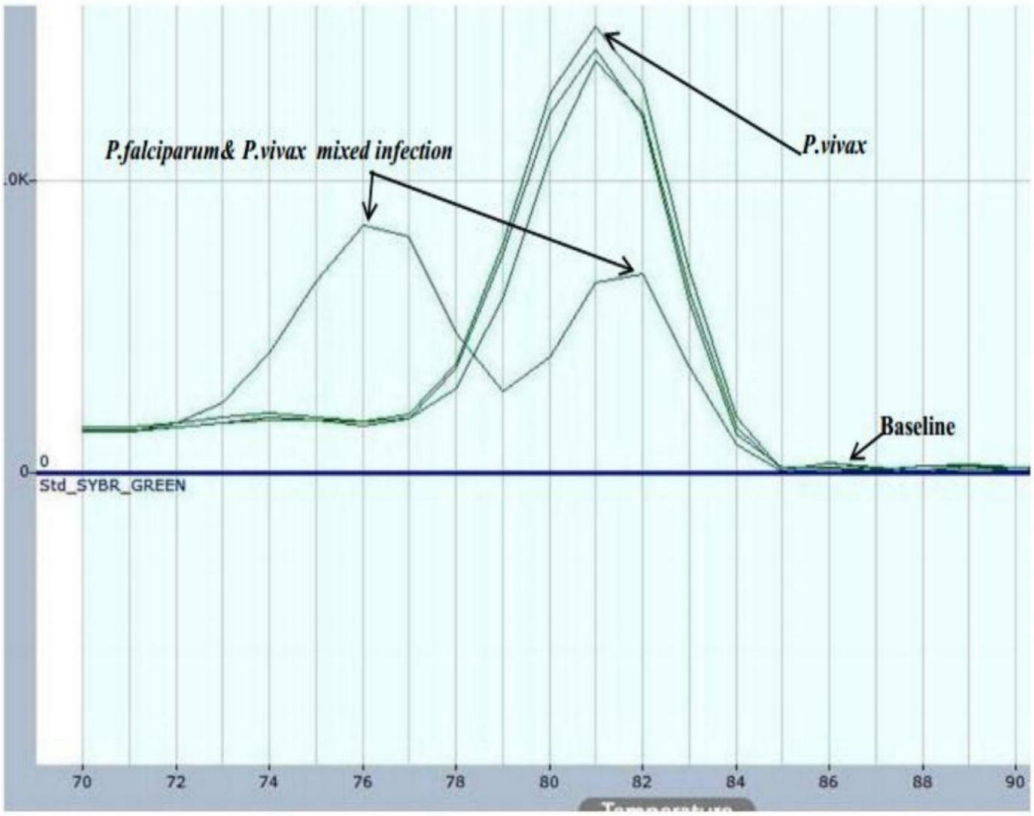

Figure 4. 10Melting curve peaks of single and mixed plasmodium species; *(Pv)* at 81^{0}C, and (*Pf)* + (*Pv)* at 76^{0}C and 82^{0}C respectively

The single infections of *Plasmodium falciparum, Plasmodium ovale* and *Plasmodium vivax* are represented at different temperature profiles and melting curve peaks .The single infections of *P.falciparum* is clustered at around at 77^{0}C. *Plasmodium ovale* single infection is lying at 79^{0}C and *P. vivax* single infection is clustered at around 81^{0}C (figure 4.11).

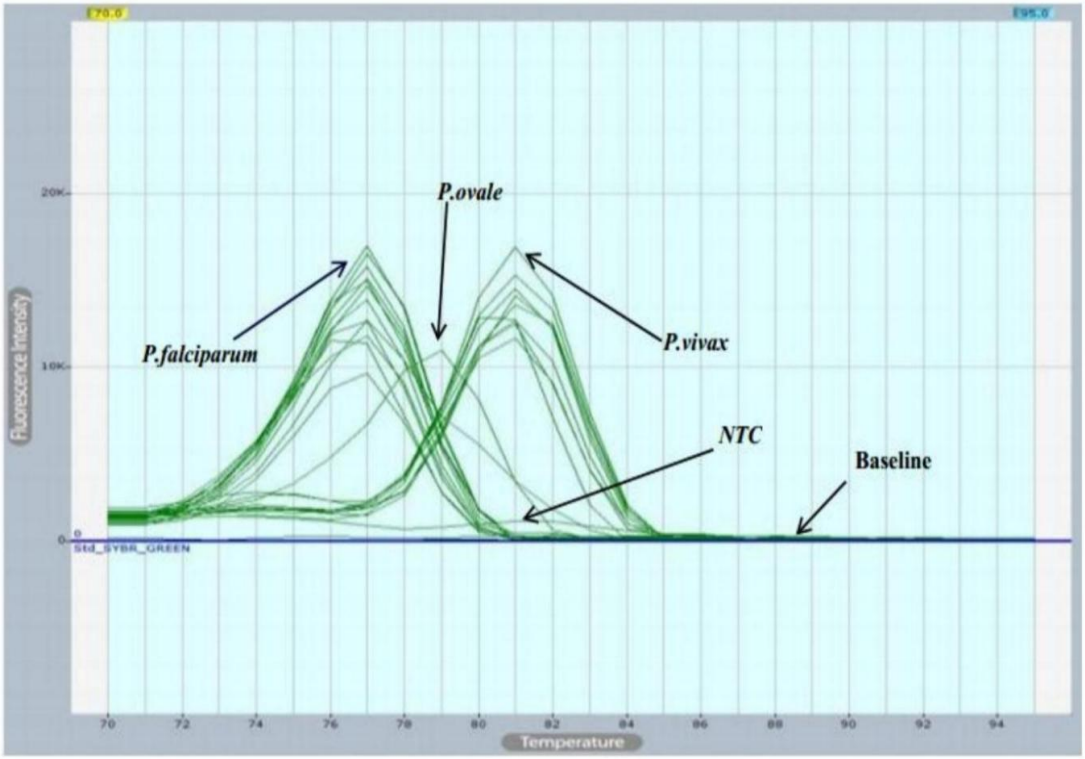

Figure 4. 11Melting curve peaks representing single *plasmodium* species; (*Pf*) at 77^0C, (*Po*) at 79^0C and (*Pv*) at 81^0C respectively

Out of the 168 dried blood spot samples 7 (4.17%) were *P. vivax* and 158 (94.04%) were *P. falciparum,* and 3 (1.79%) were both species microscopically. The molecular re-evaluation of these 168 samples by SYBR Green I based real-time PCR and melting curve analysis indicated as 112 (66.7%) *P. falciparum,*10 (5.6%) *P. vivax,* 17 (10.12%) *P. ovale* and, 21 (12.5%) *P. vivax* and *P. falciparum* mixed infections. While 8 (4.8%) were negative for any one of the malaria parasite species (table 4.1). The results indicated (27.38%) difference and disagreement between microscopy and real- time PCR. *Plasmodium ovale* was not commonly reported by microscopic diagnosis in the study area.

Table 4.1 Comparison of microscopy and real-time PCR for *Plasmodium* species detection

Parasites	Methods	
	Microscopy 168 Samples (100%)	Real-Time PCR 168 Samples (100%)
P.falciparum	158 (94.07%)	112 (66.7%)
P.ovale	0 (0%)	17 (10.12%)
P.vivax	7 (4.16%)	10 (5.6%)
P.falciparum and *P.vivax*	3 (1.8%)	21 (12.5%)
Negative	0 (0%)	8 (4.8%)

4.3.1. *Pfcrt (K76T)* identification and *Pfmdr1* (N86Y) copy number determination

In the study 112 *P. falciparum* single infections and 21 mixed infections a total of 133 samples were re-confirmed by SYBR Green I based real- time PCR as *P. falciparum* positive samples in objective one. Those samples were analysed further for *(Pfcrt, K76T)* genotyping and (*Pfmdr1*, N86Y) copy number determination.

4.3.2. *Pfcrt (K76T)* Identification

The *P. falciparum* chloroquine transporter gene was targeted for (*Pfcrt, K76T*) genotyping. The amplification of the target gene was recorded from log scale form of the amplification curves. This form of graph helps to exclude the non-specific amplifications, primer-dimers and other artefacts to the left bottom side of the graphs

(figure 4.12).The negative and positive controls were included in the experiment in each and every PCR runs.

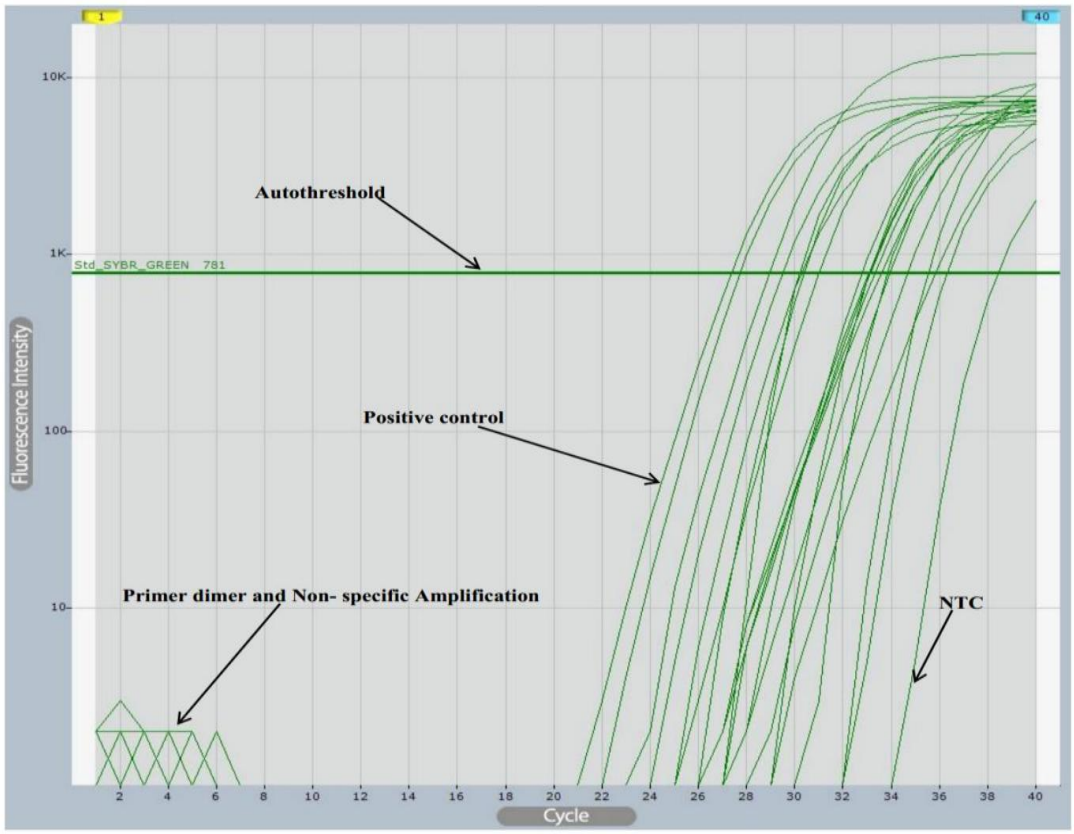

Figure 4. 12Amplification of (*Pfcrt*, K76T) in log scale form

Melting curve analysis was made for (*Pfcrt, K76T)* genotyping. The shapes of the melting curves indicated the wild type (*Pfcrt*, K76) as flat curves and mutant type (*Pfcrt*, 76T) as raised curves with dual peaks. The strains (*3D7* and *D6*) were positive controls for the wild type and showed flat curves and strain (*W2)* was positive control for mutant type and showed raised melting curve with dual peaks (figure 4.13).

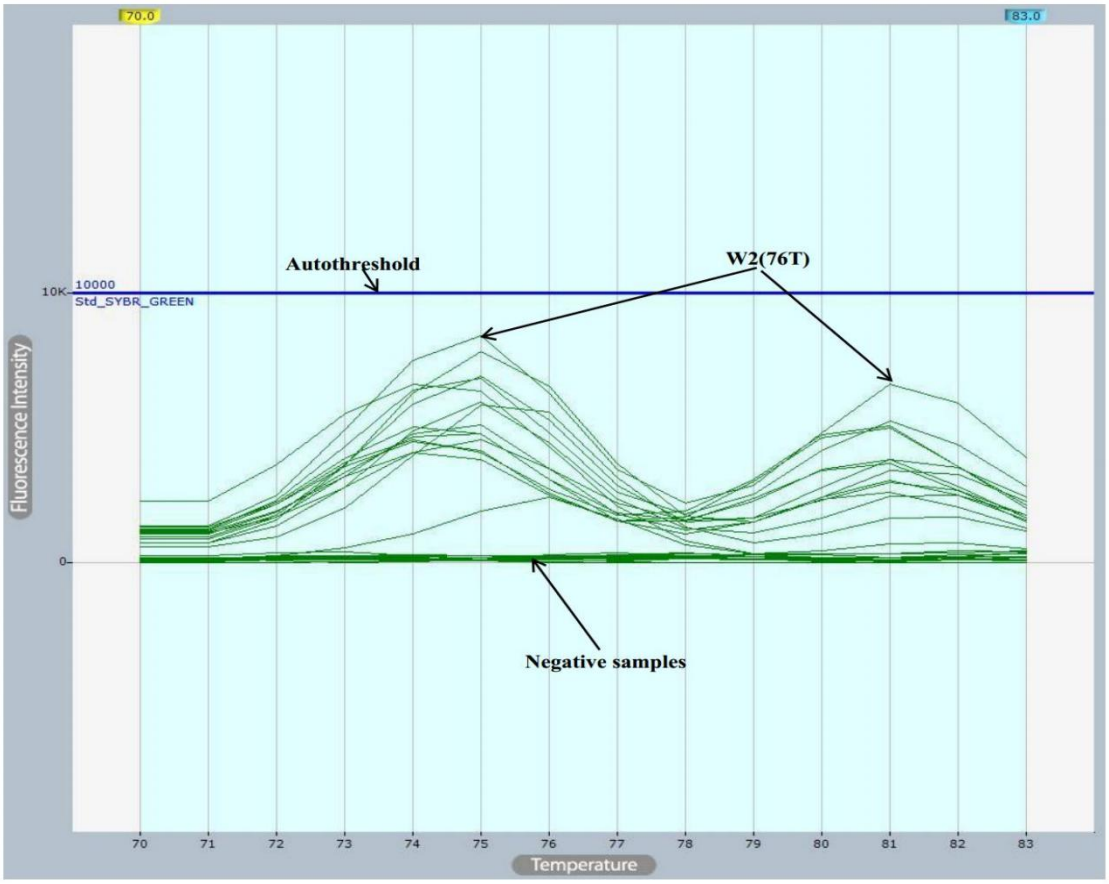

Figure 4. 13Melting curves for wild type and mutant (*Pfcrt*, K76T) gene

Among the, 133 samples 73 (54.88%) were genotyped as resistant type (*Pfcrt, 76T*) and the remaining 60 (45.11%) were genotyped as wild type (*Pfcrt*, K76). In this study higher prevalence of mutant genotype (*Pfcrt, 76T)* was found, but statistically insignificant (P=0.635, P>0.05). It was relatively higher in Kola Diba and Aymba. The other two study sites namely Makisegnit and Shedi had higher prevalence of wild type (*Pfcrt*, K76) (figure 4.14).

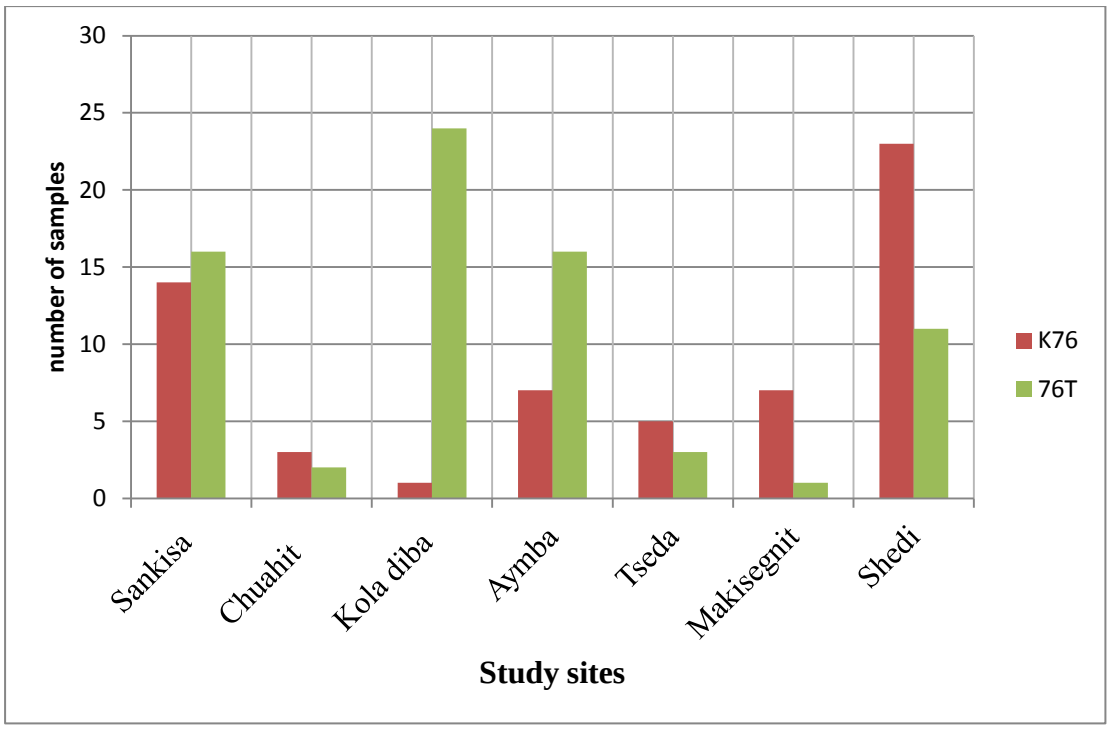

Figure 4. 14The prevalence of (*Pfcrt, K76T*) point mutations

4.3.3. *Pfmdr1* (N86Y) amplification and copy number variation

The genotyping of wild type (*Pfmdr1*, N86) and mutant type (*Pfmdr1*, 86Y) single amino acid polymorphs were done by using the dual labelled single probe, which was specifically designed to complement the mutant genotype (*Pfmdr1*, 86Y) codon mutation, and SYBR Green I dye. The yellow graphs generated by the TET-labelled probe fluorescent were represented as mutant genotype (*Pfmdr1*, 86Y) and the green graphs generated by SYBR Green I dye were represented as wild type (*Pfmdr1*, N86). The positive controls from resistant strains such as W_2 and Dd_2 were included and showed as yellow coloured graphs in the amplification graphs which confirmed as positive for mutant genotypes in the assay throughout the experiment. In contrast, sensitive strains such as *3D7* and *D6* shown as green in the amplification graph,

which confirmed as positive for wild type genotypes. Therefore, clinical samples were genotyped based on the positive controls and the colour of the graph. A few samples were categorized as the mutant genotype (*Pfmdr1*, 86Y) and the remaining of the samples were represented as wild type genotype (*Pfmdr1*, N86) (figure 4.15).

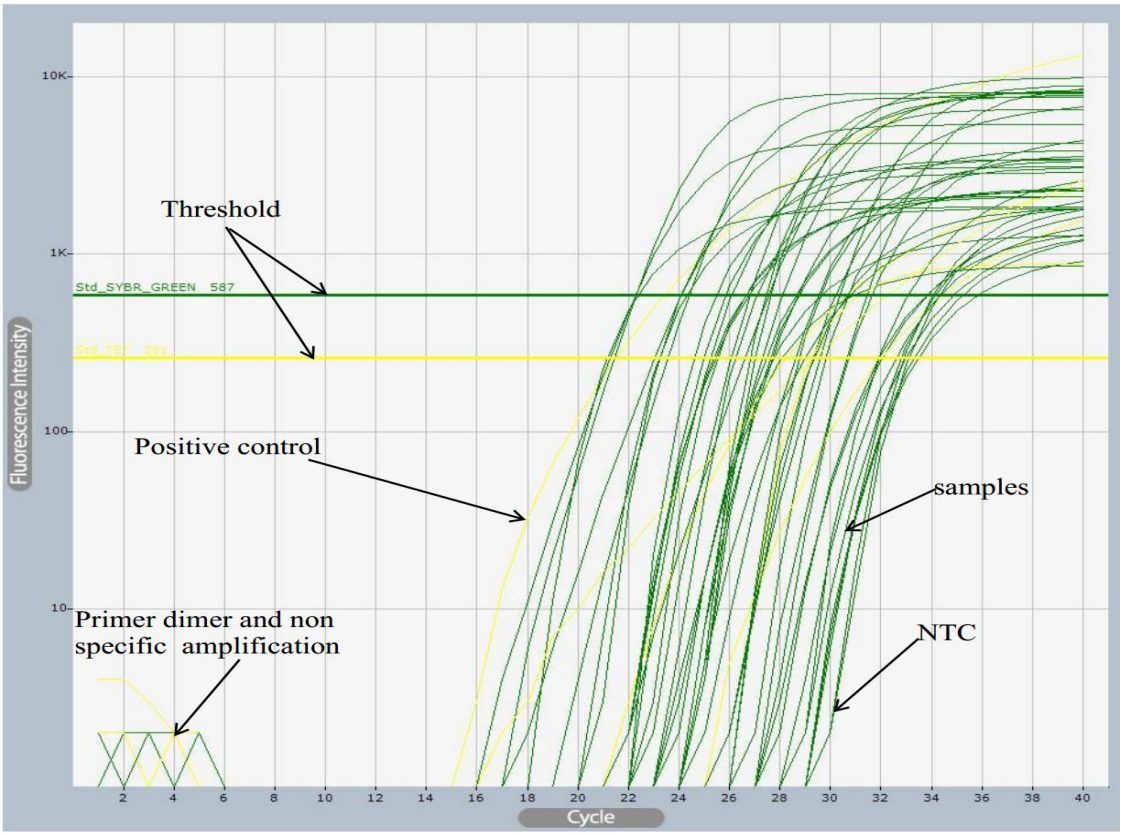

Figure 4. 15Amplification curves of (*Pfmdr1*, N86Y) gene fragment in log scale form

The melting curve analysis was not successful for further analysis of these polymorphisms. The failure of melting curve analysis could be due to loose complementationbetween the probe and the template hence it melted rapidly before formation of the sharp melt curve peaks.

In order to determine the copy number of the target gene, the standard curve was made from a known concentration of genomic DNA which was serially diluted and having the same target gene with single copy per haploid genome. Then the input DNA concentration of each clinical sample was determined by the software automatically based on the concentration of serially diluted DNA from the standard curve. The blue points on the regression line represented the concentration of the standard sample. In other words, these points were obtained from the *3D7* strain genomic DNA and the yellow points on the regression line represented the concentration of the clinical samples (figure 4.16).

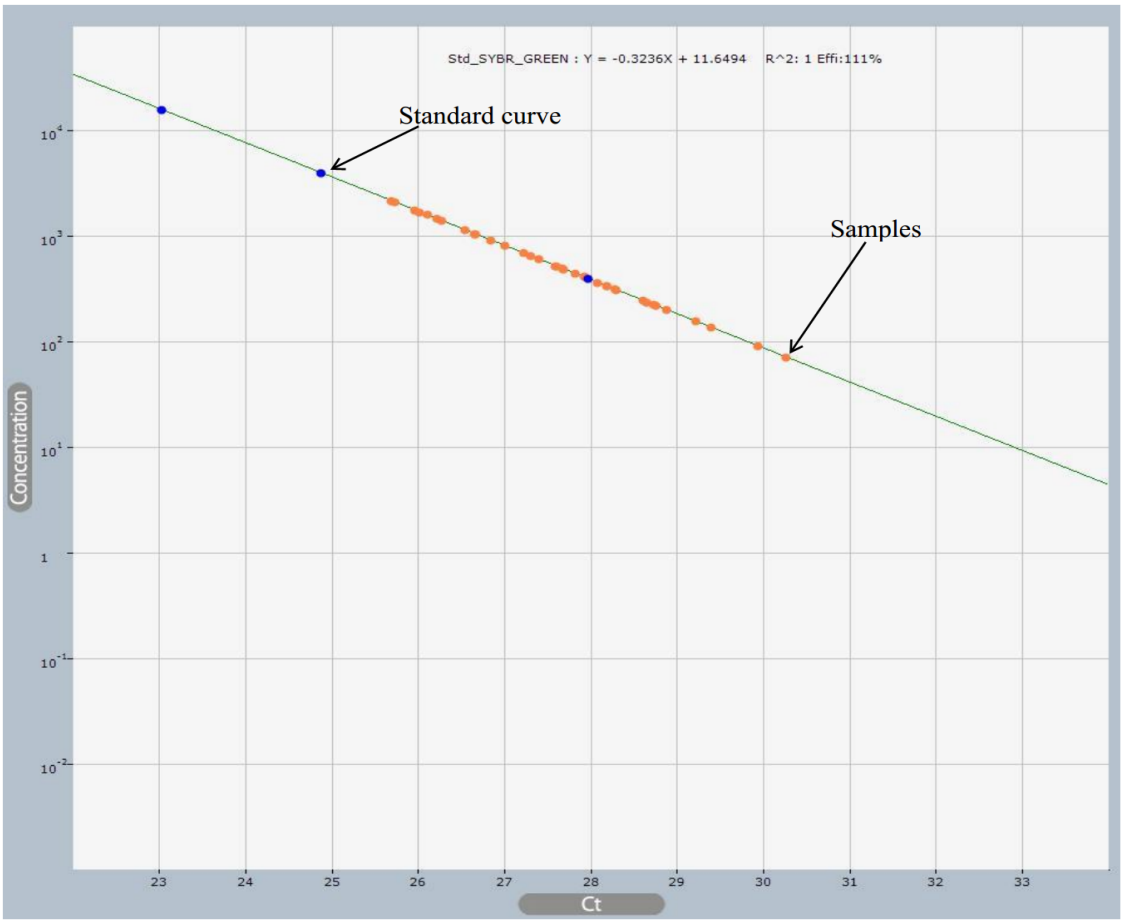

Figure 4. 16Standard curve based on serially diluted *3D7P.falciparum* genomic DNA for absolute quantification

A total of 133 samples were analysed further for determination of the (*Pfmdr1*, N86Y) copy number variation. Among the 133 samples 72(54.88%) samples were confirmed as multi-copy variants of (*Pfmdr1, N86Y*) having copy numbers of the target gene from 1.5 copies to 4.471 copies per genome and the remaining 61 (45.11%) were confirmed as single copy variants of (*Pfmdr1*, N86Y) having copy numbers less than 1.5 copies per genome (figure 4.17).

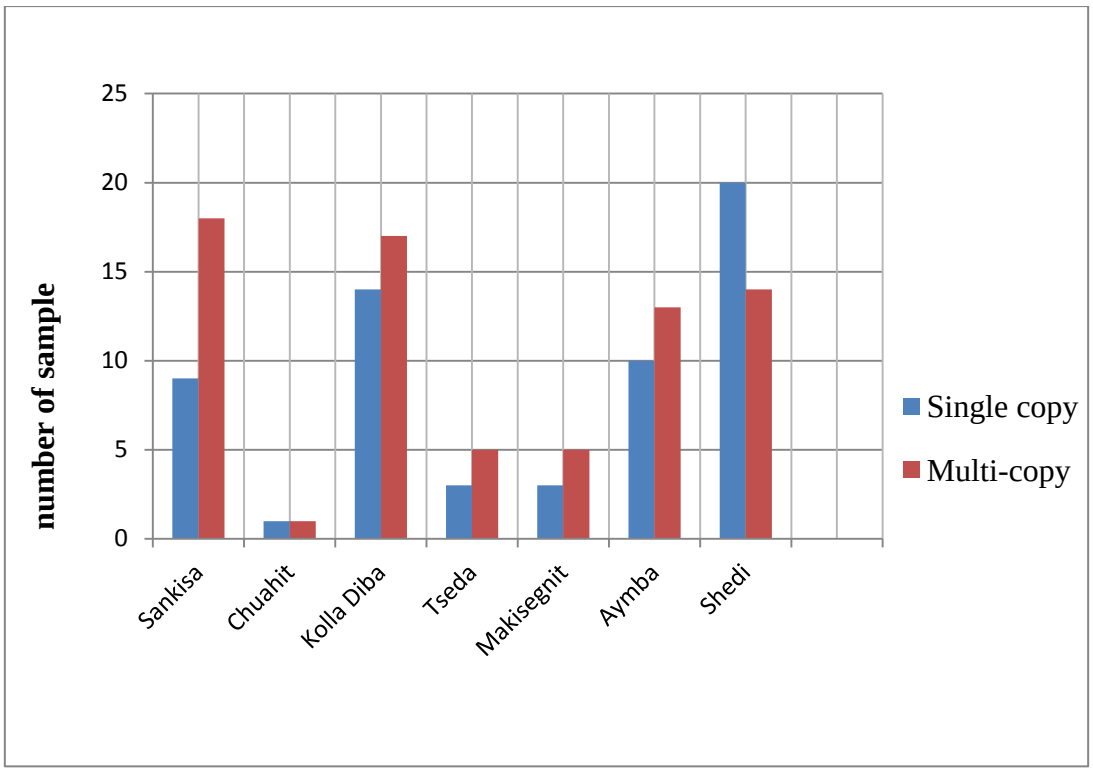

Figure 4. 17Real- time PCR detection of *Pfmdr1(N86Y)* copy number variation

The multi-copy number variants of (*Pfmdr1, N86Y*) were categorized as medium copy number variants (1.5 to 2.5 copies per genome) and higher copy number variants (2.5 to 4.741 copies per genome). In this category 34 (25.56%) were medium copy number variants of (*Pfmdr1, N86Y*) and 38 (28.57%) were with higher copy number variants of (*Pfmdr1, N86Y*) (figure 4.18).

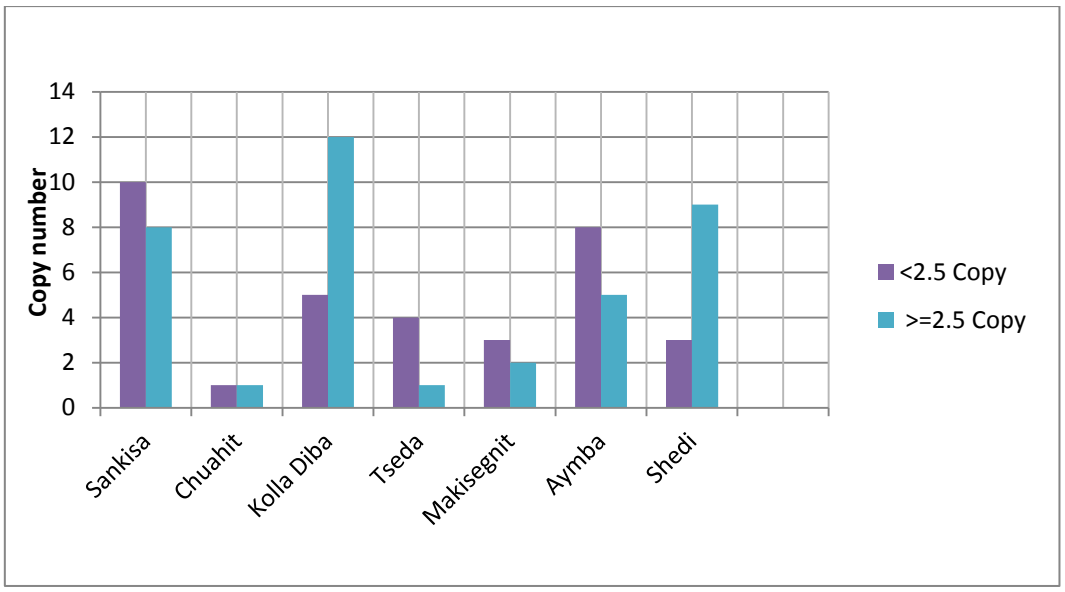

Figure 4.18 Real- time PCR detection of medium and higher copy number variants of*Pfmdr1(N86Y)*

The minimum and maximum copy numbers per genome were 0.497 (single copy) and 4.741(multi-copies), respectively. Positive controls obtained from Kenya medical research institute malaria laboratory were included in the assay. The controls were *Dd2 P. falciparum* strain which is known to have multiple copy numbers of (*Pfmdr1, N86Y*), and *W2* and *D6P. falciparum* strains which possess single copy number of *(Pfmdr1, N86Y).* The copy number variants of the positive controls were consistently found as single and multi-copy in each experimental runs.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1.Discussion

This is the first study conducted to establish mixed infection level of *P. falciparum* and *P. vivax,* and the chloroquine transporter gene point mutations at (*Pfcrt, K76T)* prevalence and (*Pfmdr1, N86Y)* copy number variation in the study area. The results obtained in this study showed high prevalence of *P. falciparum* and *P. vivax* infections as first and second prevalence, respectively. According to microscopic diagnosis record *P. falciparum* and *P. vivax* infections were the common *Plasmodia* species with first and second prevalence rate respectively in the study area (Alemu *et al*., 2012). The most malaria cases are reported due to *P. falciparum* and follows by *P. vivax*. Therefore, the prevalence rate of *P. falciparum* is usually higher compared to prevalence rate of *P. vivax*. Even though malaria transmission is heterogeneous in Ethiopia its transmission is affected by temporal and spatial factors. Malariais less in highland areas (higher altitudes) than lowland areas, but its prevalence increases from highland areas down to lowland areas (lower altitudes). *Plasmodium vivax* is more common in high altitude areas than in lower altitudes compared to *P. falciparum* (Woyessa *et al*., 2012).

Other recent study showed that *P. vivax* is second highest malaria species in malaria endemic area of Ethiopia (Kebede *et al*., 2014). Therefore, the current study showed similar results with other studies done so far on the prevalence of the two species.

Malaria incidence varies according to gender and age, with males age 5 and above showing a statistically higher incidence (Yeshiwondim *et al*., 2009). This study showed an increment of the prevalence of both species as the age increased from one to fifteen and above years. It suggested that the different exposure rate of infections with regard to age group. The infection rate of *P. falciparum* and *P. vivax* is different in females and males. Among 1,802 positive patients 80.85% were males and 19.15% were females. Males are field workers in the study area and were more infected than females which indicated that males were exposed to mosquito bite more frequently. Insecticide-treated bed nets are not commonly used by field workers in the study area, and this could explain the different rate of infections (Ouattara*et al*., 2014).

The prevalence of *P. falciparum* mono-infection was considered in all study sites to compare its prevalence in each study site. The maximum prevalence of *P. falciparum* was recorded in Metema Hospital and the minimum was recorded in Tseda clinic. Metema Hospital is found in relatively lower land than the other study sites. It provides medication for many people compared to other study sites. In addition, this hospital is found in an investment area in which seasonal labour workers come to the area where they are exposed to malaria infections.

The re-evaluation of 168 microscopically positive samples by SYBR Green I based real-time PCR melting curve analysis showed disagreement with microscopic results with regard to *P. falciparum*, *P. vivax*, *P. ovale* single infections, and *P. vivax* and *P. falciparum* mixed infections. Microscopically, misdiagnosed single infections and mixed infections of *Plasmodium* species were detected in melting curve analysis

provided that real-time PCR is sensitive and specific for clinical diagnosis. However, a few negative samples were obtained during melting curve analysis. This could be due to false positive samples during microscopy.

Plasmodium ovale was not commonly reported by microscopy in the study area. However, *P. ovale wallikeri* and *P. ovale curtisi* were reported by species specific nested PCR in Dembia district which is the same study area (Alemu *et al.*, 2013).However, in the current study *P. ovalewallikeri* and *P.ovale curtisi* were not distinguished.

The prevalence of malaria parasite infection obtained from Giemsa stain and its re-evaluation using SYBR Green I based real-time PCR method shows a high discrepancy. Real-time PCR detection of *plasmodium* species is more sensitive than microscopic diagnosis especially when the patient harbours mixed and/or a low parasitaemia (Rougemont *et al*., 2004**;** Shokoples *et al*., 2009). In this study, the SYBR Green I based real-time PCR assay for *plasmodium* species detection was more sensitive than microscopy because it was able to detect 7.9 ng/µl of target DNA concentration. A lower concentration of template DNA; less than 7.9ng/µl, has been detected, but with relatively higher Ct values. Real-time PCR detection of *Plasmodium* species by meting curve analysis is very fast and accurate compared to other molecular methods (Swan *et al*., 2005).In this study, the detection process in both amplification and melting step was approximately 2 hours and 20 minutes and *plasmodium* species were detected successfully. Melting curve analysis has an advantage to observe all *plasmodium* species simultaneously in a single step. In

addition, the method enables closed system data analysis in which it prevents Post-PCR contamination of the samples unlike gel- electrophoresis.

The results obtained in the current study assured that the use of microscopy for febrile patient diagnosis does not have reliability for effective patient treatment because microscopic diagnosis relied on morphological characterization of *plasmodium* species; hence, it depends on parasitaemia and morphological distinction of the species. However, PCR targets parts of the genome of the parasite particularly the potential variable regions of the four *plasmodia* species the18S rRNA gene, which is found as a multi-copy gene per genome. The concentration of template DNA decreases the sensitivity and specificity of PCR for *plasmodia* species detection, but the method is better compared to microscopic morphological detection sensitivity and specificity.

SYBR Green I based real-time PCR targeted *plasmodium* species 18S rRNA gene is sensitive and specific enough to detect all the four types of *plasmodium* species (Kathy *et al*., 2005; Taylor *et al*., 2011)**.** Single pair of consensus species-specific primers targeted at variable regions of the *plasmodium* species multi-copy 18S rRNA gene amplifies the template DNA from 18S rRNA gene of *plasmodia* species and the melting curve analysis separates these *plasmodia* species based on melting temperature profile, and melting curve shapes and peaks at species level (Kathy *et al*., 2005). In this study, the same primer was used and *plasmodia* species were successfully detected. The melting curve analysis separates any PCR product based on the amplicon length and its G+C content. The average G+C content of *P. vivax* and

P. falciparum is 42.3% and 19.4% respectively (Carlton *et al*., 2008). Therefore, *P. vivax* has relatively higher temperature profile than *P. falciparum.* As a result, all the three *plasmodium* species; *Plasmodium ovale, Plasmodium falciparum, Plasmodium vivax* and mixed infections of both *P. falciparum* and *P. vivax* were accurately detected. However, mixed infections of *P. ovale* either with *P. falciparum* or *P. vivax* was not detected in this study. The results obtained in this study agree with above findings. Therefore, the use of SYBR Green I based real-time PCR with melting curve analysis step is applicable for effective malaria species diagnosis. Misdiagnosis of malaria parasites lead to mistreatment of patients with wrong drug prescription that pose great problem for emergence of new drug resistance malaria species, economic loses and treatment failure **(**Kebede *et al*., 2014**).** Furthermore**,** fixations of previous mutations occur due to misdiagnosis and mixed infections. The study found higher prevalence of mixed infections, misdiagnosis and fixation of the mutant (*Pfcrt, 76T)* mutation. The copy number variations in the (*Pfmdr1, N86Y*) gene was high, but statistically it was not significant (P= 0.455, P>0.05).

The mutant (*Pfcrt,* 76T) point mutation is the marker which shows the status of chloroquine resistant *P. falciparum*. The high prevalence level of this mutation is an indicator for the spread of drug resistant *P. falciparum* population in the study area. Even though, there are no related data done for the prevalence level of chloroquine resistant *P. falciparum* in the study area for comparison the result obtained from this study showed high prevalence of mutant (*Pfcrt, 76T),* but statistically not significant (P=0.635, P>0.05). The mutant (*Pfcrt, 76T*) point mutation is a well-established molecular marker for chloroquine resistance level and prevalence rate of the resistant

parasite because (*Pfcrt, 76T)* is the first point mutation to occur and shows high level of resistance compared to the rest of the point mutations at *Pfcrt* gene (Gadalla *et al*.,2010). Therefore, the higher the prevalence of this mutation, the more risk of malaria and challenging for drug resistant *P. falciparum* malaria control. On the one hand, high prevalence of mutant (*Pfcrt, 76T)* suggested that the presence of drug pressure or other factors which favours (*Pfcrt, 76T)* mutation.

Chloroquine was incompletely withdrawn from the study area since 2004 for treatment of *P. falciparum* malaria (Ambachew, 2012), and the parasite population with (*Pfcrt, 76T)* point mutation was expected that it would have been reduced on the 10 years period. Replacement of chloroquine with other drug for *P. falciparum* treatment might disadvantage the mutant parasite survival. However, the parasite with mutant (*Pfcrt, 76T*) genotype was fixed. The following reasons are contributing factors for fixation of (*Pfcrt, 76T*) mutation. Firstly, incomplete withdrawal of chloroquine in the study area. Chloroquine is still in use for treatment of *P. vivax.* Secondly, high prevalence of mixed species infections.*Plasmodium falciparum* and *P. vivax* are endemic to the study area; therefore, mixed infection is common. Thirdly, poor clinical diagnosis. The facilities and the skills of the laboratory technicians are critical for accurate detection of *plasmodia* species. The fourth is poor management of malaria drugs. Patients purchase drugs from the shop or local pharmacy and treat themselves without clinical diagnosis confirmation (Alemu *et al*., 2012).

If chloroquine was completely withdrawn, the parasite would have reverted to wild (*Pfcrt, K76*) and chloroquine could be used for treatment of *P. falciparum*. Studies in

Kenya, Tanzania and Malawi showed the conversion of mutant (*Pfcrt, 76T*) to wild (*Pfcrt, K76*) and reduction of mutant (*Pfcrt, 76T*) prevalence (Mwai *et al.*, 2009; Frosch *et al*., 2011). The use of artemesinin combination therapy (ACT) has increased throughout Africa (Fançony *et al*., 2012). Therefore, the prevalence of (*Pfcrt, 76T*) point mutation was expected to be reduced in Africa. However, in Southern Ethiopia one study showed that high prevalence of (*Pfcrt*, K76T) and (*Pfmdr1*, N86Y) point mutations (Schunk *et al*., 2006). The drug chloroquine could be used again as combination or single treatment drug to treat malaria caused by *P. falciparum* where chloroquine mutation prevalence is very low or has disappeared (Gadalla *et al*., 2010). The current study and another study done in Ethiopia (Schunk *et al*., 2006) showed a high prevalence level of chloroquine resistant *P. falciparum* point mutation, and hence re-use of chloroquine for *P. falciparum* treatment in the Ethiopian context is disadvantageous.

Malaria parasite population dynamically evolves along with anti-malarial drug pressure. The change of treatment pressure or drug affects the parasite evolutionary process. The malaria drugs can be reused after a long period of withdraw. Both *Pfcrt* and *Pfmdr1* have synergic effect on chloroquine resistance level. The prevalence of *P. falciparum* with (*Pfcrt, 76T*) point mutation and high copy number variability at (*Pfmdr1, N86Y)* gene could trigger resistance for the new drug coartem and impair the re-introduction of the previous drugs in the study area. The high prevalence of (*Pfcrt,*76T) point mutation with either *Pfmdr1*(N86) or *Pfmdr1*(86Y) high copy can affect the treatment of malaria.

In this study high prevalence of *Pfmdr1(N86Y*) copy number variants were found, but it was not significant statistically (P=0.455, P>0.05). Copy number variation exists among *P. falciparum* strains regardless of codon mutation. Parasites do have coping mechanism against drug pressure and the mechanism is highly dynamic across *P. falciparum* population. When chloroquine was a treatment drug of choice for *P. falciparum* the parasite was able to survive and become fully resistant to it. Codon mutation and/or copy number variation is/are the most common means of resistance development against the most treatment drugs. Studies showed that copy number variations occur first and the *P. falciparum* becomes tolerant for many drugs. The higher the copy numbers of (*Pfmdr1*, N86Y) gene is, the more the tolerance against treatment drugs. Even the uses of combination drugs become risky for effective treatment. High population of multidrug resistance mutation and copy number variation in *P. falciparum* develop resistance for many drugs like mefloquinine, lumefantrine and/or artemisinin derivative combination drugs such as coartem (artemether/lumefantrine).

An *in vitro* study done in Ghana showed that nascent clones of *P. falciparum* manifested by reduced susceptibility to artemisinin derivatives (Duah *et al*., 2013). Since lumefantrine and mefloquinine have physiochemical similarity, therefore, resistance to mefloquinine can be modulated by lumefantrine. A study done in Kenya showed that (*Pfmdr1, N86Y)* has association with mefloquinine response (Eyase *et al*., 2013). Another *in vitro* study in Cambodia-Thailand border showed that parasites with higher copy number showed significantly reduced susceptibility to mefloquinine,

lumefantrine and artesunate. However, *Pfmdr1* polymorphism showed no relationship in *in vitro* susceptibility of these drugs (Lim *et al*., 2009).

The *Pfmdr1* copy number variation is more frequent event than *Pfmdr1* codon mutation (Preechapornkul *et al*., 2009). A knockdown study done on *Pfmdr1* gene copy number showed reduced susceptibility to MFQ, ART, LUM in an *in vitro* susceptibility test. In contrast, this knockdown study showed that parasite line with low copy numbers had heightened susceptibility to ART and LUM (Sidhu *et al*., 2009). Parasites with single copy number are sensitive for mefloquinine, lumefantrine and artesunate. These drugs can be used for combination therapy where the population of *P. falciparum* with single copy number of *Pfmdr1* gene are fixed (Khattak *et al*., 2013).

The study of copy number variation in *P. falciparum* population is very vital for monitoring of drug resistant *P. falciparum* malaria particularly where mefloquine and lumefantrine are used for combination therapy (ACT). In the Ethiopian context coartem is the treatment drug for uncomplicated malaria especially for *P. falciparum* treatment. Since coartem is an ACT drug which has lumefantrine component, the presence of strains with high copy number variation in *P. falciparum* seems inevitable. The current study found high prevalence of multi-copy number variants in the study area. The parasite shifts from high *Pfmdr1* copy number to low copy number or vice versa depending on the drug pressure in the parasite population. *P. falciparum* with higher copy number is spread and favoured when mefloquinine, mefloquinine combination or drugs physiochemical similarities to mefloquinine are

used for treatment of *P. falciparum*. In contrast, *P. falciparum* populationwith higher copy shift to population of parasites with low copy because withdrawal of mefloquinine pressure has fitness disadvantage for continuation of mefloquinine resistant *P. falciparum* (Preechapornkul *et al*., 2009).

In this study a high prevalence of *P. falciparum* with a higher *Pfmdr1* copy number variants were found. This showed that fixation of the *P. falciparum* population with survival advantage with possessing multiple copy of (*Pfmdr1, N86Y)*. It is, indirectly, an alert that there is drug pressure which favoured these populations in the study area. The reasons could be incomplete withdrawal of chloroquine, use of lumefantrine combination drug (coartem), misdiagnosis of single infections and mixed infections. The high prevalence of mixed infection and misdiagnosed *P. falciparum,* which would have been treated by undesired drug, could have contributed to fixation of the resistant *P. falciparum*.

5.2. Conclusions

The results showed that malaria is a public health concern in the study area. It indicated a high percentage difference of misdiagnosed malaria species as single and mixed infections between microscopy and real-time PCR. These discordant or mismatched results obtained from microscopy and SYBR Green I based real-time PCR amplification showed that clinical diagnosis of malaria species and detection of mixed species needed special concern in the study area. Although it is not statistically significant, a high prevalence of chloroquine resistant *P. falciparum* obtained in (*Pfcrt*, K76T) point mutation showed fixation of previous chloroquine resistant *P.*

falciparum population after incomplete withdrawal of chloroquine in the study area. The prevalence of (*Pfmdr1*, N86Y) copy number variant was not statistically significant, but it was high and found to be an indicator of the evolutionary path shift from resistant *P. falciparum* for monotherapy to resistant *P. falciparum* population against combination drug therapy. Therefore, a high copy number variation is shown to be a risk factor for use of combination drugs for treatment of malaria.

5.3.Recommendations

The study recommends the use of real-time PCR for accurate detection of single and mixed infections of malaria species. This is very important for adherence of proper treatment regimen and detection of emergency of drug resistant strains. Further, national studies should be done to determine the prevalence level of *P. falciparum* and *P. vivax* species misdiagnosis and mixed infections and monitor development of resistance parasite populations. It is helpful to manage fixation and spread of drug resistant *P. falciparum* malaria. In order to determine continuation or discontinuation of chloroquine use the prevalence level of *P. falciparum* chloroquine resistance point mutations and its fixation should be analysed. Comprehensive molecular surveillance is needed in order to control the spread of *Pfmdr1* copy number variants in *P. falciparum* population and which could be useful indicator of resistance development against combination therapy because *Pfmdr1* copy numbers are increased when mefloquinine and related drugs are used for treatment of *P. falciparum.*

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APPENDICES

Publication

1. Tajebe, A., Magoma, G., Mulugeta, A. and Kimani, F.(2014). Detection of mixed infection level of *P. faciparum* and *P. vivax* by SYBR Green I based real-time *PCR .Malar J.13*:411.DOI:10.1186/ISSN 1475-2875-13-411.

Ethical clearance

Ethical clearance obtained to conduct the study

Consent form

Consent form for study participants

University of Gondar
Faculty of Natural and Computational Sciences
Gondar, Ethiopia

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Ref: No FNCS/10/ 2069 / 2013
Date 24/10/2013

To whom it may concern

Subject : ***Ethical clearance***

A research project entitled " Detection of P. falciparum and P. vivax Coinfection level and Identification of Chloroquine Resistance Mutations in Chloroquine Transporter genes of P. falciparum in Dembia plain, Ethiopia "has been reviewed by the ethical committee of the Faculty of Natural and computional Science and found to ethically acceptable. Thus, the committee has authorized the principal investigator **Mr. Admas Tajebe** to commence his project accordingly.

The investigator is expected to strictly follow and fulfill the following obligations and responsibilities.
The investigator:-

1. Should comply with the standard national and international scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs approval of the ethical review committee of the Faculty of Natural and computional Science.
3. The principal investigator should submit follow up report every month until completion.
4. The final thesis work should be reported to the Faculty of Natural and computional Science ethical committee.

Chair person
Name Dr. Beyhanu Andualem
Sig.
Date 24-10-2013

Faculty dean
Name Getnet A
Sig.
Date Oct 24, 2013

Faculty of Natural & Computational Science University of Gondar

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Annex A. Consent Form

The study will be conducted under the objective of Identification of *P. falciparum* Chloroquine Resistance Transporter Gene and Detection of *P. falciparum* and *P.vivax* Coinfection level in Dembia district. A trained health professional will take a blood sample from selected and volunteer study participants by finger pricking. To avoid infections with blood born pathogens like HIV, one disposable lancet will be used for finger pricking for each study participants. For those who have bleeding problem, special care will be given. All costs related anti- malaria drugs (if the study participants became positive) will be covered by the project. Study participants have a right to withdraw from a project if they feel discomfort from the purpose of study or any personal reasons. If a study participants or any responsible body have any questions about the project please contact with principal investigator Ato Addimas Tajebe (Phone number 0920618229) or University of Gondar Biology Department.

I, who registered in--1140---------- identifications number, clearly understood the above statement and agree to participate in the study.

Name and signature of the participant / parent/ care taker

Ayele Kebede _____

Annex B. Assent Form

This study will be conducted for the purpose of Identification of *P. falciparum* Chloroquine Resistance Transporter Gene and Detection of *P. falciparum* and *P.vivax* Coinfection level in Dembia district. A blood sample will be collected by finger pricking from children also. To avoid infections with blood borne pathogens like HIV, one disposable lancet will be used for finger pricking for each child. For those who have bleeding problem, special care will be given. All costs related to anti- malaria drugs (if the study participants became positive) will be covered by the project. If the children, parents or guardians are not conformable to the study they have right to withdraw from the study. If a study participants or any responsible body have any questions about the project please contact with principal investigator Ato Addimas Tajebe (Phone number 0920618229) or University of Gondar Biology Department.

I, who registered in--1174---------- identifications number, clearly understood the above statement and agree to participate in the study.

Name Selamawit Molla ---------- signature ----------