"Chloroquine Resistance Transporter (*pfcrt*) and Multidrug Resistance 1 (*pfmdr1*) Genes Mutation in *Plasmodium falciparum* Population under Varying Level of Endemicity with *Plasmodium vivax* in Selected Parts of Ethiopia."

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DECLARATION

I here declare that this thesis is my original work and that all source of materials used for this thesis have been fully acknowledged. This thesis is submitted to Addis Ababa University, Institute of Biotechnology in Partial Fulfillment of the Requirements for the Masters of Science Degree in Biotechnology, Addis Ababa, Ethiopia.

I truly declare that this thesis has not been submitted to any other University anywhere for the award of any academic degree.

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Lists of Abbreviation

ACT	Artemisinin Based Combination Therapy
AHRI	Armauer Hansen Research Institute
ART .	Artemisinin
CNV	Copy Number Varation
CQ	Chloroquine
CSA	Central Statistical Agency
CT	Threshold cycle
DBS	Dried Blood Spots
DNA	Deoxyribonucleic Acid
DV	Digestive Vacuole
FAM	Fluorescein Amidites
FMOH	Federal Minister of Health
GCN	Gene Copy Number
GMS	Greater Mekong Subregio
Hb	Hemoglobin
HC	Health Centers
HSTP	Health Sector Transformation Plan
IPT	Intermittent Preventive Therapy
IRS	Indoor Residual Spraying

LLINs	Long-Lasting Insecticide-Treated Nets
PfCRT	*Plasmodium falciparum Chloroquine Resistance Transporter*
PFMDR1	*Plasmodium falciparum multi drug resistance 1*
PfMRP	*Plasmodium falciparum Multidrug Resistance Protein*
PfNHE1	*Plasmodium falciparum* Na^+/H^+ Exchanger 1
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SEA	Southeast Asia
SSA	Sub-Saharan Africa
TAMRA .	Tetra Methyl Rhodamine
VIC	Victoria
WHO	World Health Organization

Abstract

The global controlling of *Plasmodium falciparum* infections faces significant challenges due to the spread of parasites resistant to antimalarial drugs. In Ethiopia, where both *P. vivax* and *P. falciparum* coexist, the treatment for uncomplicated *falciparum* malaria shifted from chloroquine (CQ) to sulfadoxine-pyrimethamine (SP) in 1998 and then to Coartem (artemether-lumefantrine (AL)) in 2004. AL has been the standard treatment for over two decades for *P. falciparum*, while *P. vivax* is still treated with *CQ*. The coexistence of these two species and the accessibility of *CQ* for *P. vivax* treatment raise questions about whether switching from *CQ* to *AL* for *P. falciparum* treatment might lead to the resurgence of CQ - susceptible *P. falciparum* strains due to reversal mutations or the efficacy of *AL*. The sudy aimed to assess the prevalence of *pfcrt-76* and *pfmdr1-86* gene mutations in the *P. falciparum* population under varying levels of endemicity with *P. vivax* in selected parts of Ethiopia. In this study the frequency of gene mutations of *P. falciparum chloroquine resistance transporter 76 (pfcrt-76) and P. falciparum multidrug resistance 1-86* (*pfmdr1-86)* in *P. falciparum* collected from malaria-infected patients in Abobo, Dera, Fentale, and Metema districts, Ethiopia, were examined. Confirmed *P. falciparum* samples (n = 258) with microscope were collected through health facility-targeted cross-sectional surveys in areas with varying levels of *P. falciparum* and *P.vivax* prevalence. Genomic DNA was extracted from dried blood spots by using MagMAX DNA Multi-Sample Kit, operated by the Kingfisher Flex Automated Extractor. An 18S rRNA gene-based multiplex real-time PCR assay was employed to confirm *P. falciparum* mono-infection samples (n = 258). The study analyzed 258 *P. falciparum*-infected patients using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), examining *Pfmdr1-86* and *Pfcrt-76* gene mutations. Fisher's exact test determined marker distribution significance. Out of 250 genotyped for *Pfcrt K76T*, 68.8% had mutant 76T, and 31.2% were wild-type K76. For 257 genotyped for *Pfmdr1-N86Y*, 98.44% was N86 wild-type, and 1.56% was 86Y mutants. The mutant *Pfcrt-76T* was more prevalent in areas with higher *P. vivax* endemicity, including 93.33% (Fentale), 84.71% (Dera), 57.5% (Metema), and 43.75% (Abobo). *Pfcrt-76* gene single nucleotide polymorphisms (SNPs) significantly correlated with *P. vivax* endemicity (P = 0.000). *Pfmdr1-N86* was predominantly 100% except in Abobo, where 95.18% were N86, and 4.81% were 86Y. *Pfmdr1* gene SNPs were significantly associated with *P. vivax* endemicity (P = 0.025). Despite *CQ* discontinuation for over two decades in Ethiopia, a substantial proportion of *P. falciparum* isolates still carry mutant 76T genotypes, indicating latent *CQ* pressure. The use of *AL* for uncomplicated *P. falciparum* malaria may lead to the return of *Pfmdr1* N86 wild-type genes. Further molecular epidemiological investigations in varied endemic regions with different *CQ* usage histories are recommended to understand *chloroquine (CQ)* susceptibility recovery and AL therapy efficacy.

Keywords: *Plasmodium falciparum,* Drug resistance, *pfcrt K76T*, *pfmdr1-N86Y*, Artemether-lumefantrine, Chloroquine resistance, Chloroquine sensitive, Ethiopia.

1. Introduction

1.1. Background

Malaria globally poses threats to public health, with a disproportionately higher burden of the disease in Sub-Saharan Africa (SSA) (WHO, 2017). The World Health Organization (WHO) reported that in 2018, there were 2.4 million estimated cases among the 109 million people living in Ethiopia (WHO, 2019). There were 8,041 deaths and 2,783,816 cases in 2021 in the country (Werissaw Haileselassie et al., 2023; WHO, 2022). *Plasmodium falciparum* (~ 60%) is responsible for the higher burden of the disease in the country, followed by *P. vivax* (~ 40%) (Hiwot S et al., 2018).

The WHO's recent annual global malaria report estimates that 229 million cases of malaria were reported in 2019 in 87 countries where malaria is common. This is a 9 million case reduction since 2000. Nonetheless, it exceeded the two hundered eighteen million estimated malaria cases reported in the Malaria Global Technical Strategy, 2016–2030 (WHO, 2020a). This is reflected in the worldwide case incidence of malaria (number of cases per 1000 at-risk individuals) dropped from 80 in 2000 to 58 in 2015 and 57 in 2019.

The deployment of early diagnosis, effective treatment, and integrated vector management are primarily to blame for the decline in malaria death and incidence rates in Ethiopia (Berhane *et al*., 2011). The main method of treating malaria is still chemotherapy, and modern approaches to treating malaria rely on the administration of pharmacological combinations that contain artemisinin molecules, known as artemisinin combination therapy (ACT). Artesunate-mefloquine, artemether-lumefantrine (AL), artesunate-sulfadoxine-pyrimethamine, artesunate-amodiaquine, and Dihydroartemisinin-piperaquine (DHA-PPQ) are now the five artemisinin-based combinations that the WHO recommends (WHO, 2015). However, the development of strains of *P. falciparum* resistant to first-line antimalarial drugs has significantly complicated efforts to combat malaria going forward (Burrows et al., 2011). The WHO's drive to eradicate the illness is focusing on overcoming drug-resistant malaria since it is causing a rise in malaria cases and fatalities (Dhorda *et al*., 2021).

Anti-malarial resistance to drugs poses a global danger to the control and eradication of malaria and is especially worrisome for *P. falciparum*, the most lethal human parasite type (Commons *et al*., 2017). The rapid spread of malaria and the development of resistance to

chloroquine (CQ) against uncomplicated cases of malaria are major concerns in the treatment, control, and eradication of the disease worldwide (Huang *et al*., 2016; Some *et al*., 2016). Recent reports of parasites that are drug-resistant in Africa are signs that a new challenge is emerging for malaria management and eradication efforts (Uwimana *et al*., 2020; White, 2021).

Failures of ACT treatment are associated with the selection of certain genotypic variations of parasites, including *pfmdr1 N86* and *pfcrt K76* (Malmberg *et al*., 2013). Resistance to CQ has linked to the *pfmdr1* gene. Reducing parasite sensitivity to different drugs, such as ACT, is achieved by single nucleotide polymorphisms (SNPs) at codons 86 (N86Y), 184 (Y184F), and 1246 (D1246Y) (Holmgren *et al*., 2007). For instance, when the *pfcrt T76* genotype is paired with the *pfmdr-1 Y86* mutation, high-level CQ resistance is linked to it (Duraisingh and Cowman, 2005). Chloroquine T76 mutations are linked to resistance (Fidock *et al*., 2000), whereas its degree may be modulated by a Y86 variant of the multidrug resistance analog (*pfmdr1*) (Sanchez and Lanzer, 2000). Thus, the sudy aimed to assess the prevalence of *pfcrt-76* and *pfmdr1-86* gene mutations in the *P. falciparum* population under varying levels of endemicity with *P. vivax* in selected parts of Ethiopia.

1.2. Statement of the Problem

Drug-resistant malaria is resulting in a revival of malaria incidences and deaths (Dhorda *et al*., 2021). In 1996, reports of *P. falciparum* treatment failure in Ethiopia were released (Tulu *et al*., 1996). The country changed its treatment approach for uncomplicated falciparum malaria from CQ to sulfadoxine-pyrimethamine (SP) in 1998 due to the increasing prevalence of *P. falciparum* that is resistant to CQ. In 2004, AL replaced SP. However, CQ was kept in place and is still used as a first-line treatment for vivax malaria (Lemu Golassa et al., 2015; Elifaged Hailemeskel et al., 2019; Jifar Hassen et al ., 2022). AL efficacy was 100%, according a nationwide study carried out in 2004—the year ACT was replaced (T Kefyalew et al., 2009). But according to recent research, Coartem's susceptibility to the parasite *P. falciparum* has decreased or it has developed resistance (Abdulhakim Abamecha et al., 2021; Woticha, 2016). Failures of treatment with ACT are associated with the selection of certain wild-type parasite genotypic variations, such as *pfcrt-76* and *pfmdr1-86* (Malmberg *et al*., 2013). Numerous investigations have shown an excellent association between the mutant alleles (*pfmdr1-Y86* and *pfcrt76T*) with a high level degree of resistance to chloroquine (Seleshi Kebede et al., 2014; Ndam *et al*., 2017). It's uncertain if treating *P. falciparum* with AL instead of CQ has any indications about the efficacy of AL or is causing a repopulation of chloroquine-susceptible wild-type *Pfmdr1-N86* and *Pfcrt-K76* genes due to a reversal mutation (Lemu Golassa et al., 2015; Elifaged Hailemeskel et al., 2019; Jifar Hassen et al ., 2022).

Thus, continuous CQ usage may result in noticeably divergent selection pressure and, consequently, diverse drug resistance profiles in communities where *P. falciparum* and *P. vivax* are co-endemic (Elifaged Hailemeskel et al., 2019). The aim of the current study was to ascertain the prevalence of *Pfcrt 76* and *Pfmdr1-86* gene mutations in the study population, which was selected from the districts of Fentale, Metemma, Dera, and Abobo during the study period (from July 2021 to October 2021).

1.3. Significance of the Study

In Ethiopia, malaria continues to be a serious public health issue (WHO, 2018). It is clear that the overall status of malaria across the world is the total status of every single country, and that every single country's position is the complete status of all of its local and regional circumstances at every level. Accurate assessment of the prevalence of *pfmdr1-86* and *pfcrt-76* gene mutations in malaria burden is crucial to achieving the country-wide goal of nationwide malaria elimination by 2030 (FMOH, 2017). So, information on the status of *pfmdr1-86* and *pfcrt 76* gene mutations is critical to tailor interventions for designing effective treatments that tackle the parasite resistance raised due to *pfmdr1-86* and *pfcrt 76* gene mutations and for monitoring malaria in a particular area and possibly modifying malaria control measures in the malaria elimination campaign. In view of this, several research have been carried out and are still being undertaken in various parts of Ethiopia. This study will offer information about the parasite population that is now present in the study sites, and profiling the chloroquine markers will give information about whether there has been a change in the genetic makeup of these markers as a result of a change in the treatment strategy.

1.4. Scope of the Study

The scope of the study was limited to the prevalence of *pfmdr1-86* and *pfcrt-76* gene mutation surveillance in Fentale, Metemma, Dera, and Abobo districts from July 2021 to October 2021.

1.5. Objective of the Study

1.5.1. General Objective:

To assess the prevalence of *pfcrt-76* and *pfmdr1-86* gene mutations in the *P. falciparum* population under varying levels of endemicity with *P vivax* in selected parts of Ethiopia.

1.5.2. Specific Objectives:

- ✓ To determine the prevalence of *pfmdr1-86 and pfcrt76* genes point mutations using the nested PCR-RFLP technique.
- ✓ To analyze the distribution of *pfmdr1-86* and *pfcrt76* genes point mutations under varying levels of endemicity with *P. vivax*.

1.5.3. Research Questions

1. How prevalent are the *pfmdr1-86* and *pfcrt-76* genes in the study areas?

2. Is there a significant difference in *pfmdr1-86* and *pfcrt 76* gene mutations in areas that are *P. falciparum* dominant areas versus high *P. vivax* co-endemic areas?

1.5.4. Alternative Hypothesis (Ha)

There is a significant difference in *pfmdr1-86* and *pfcrt76* gene mutations in areas that are *P. falciparum* dominant versus high *P. vivax* co-endemic areas.

2. Literature Review

2.1. Malaria as a Major Public Health Problem

Malaria is one of the top causes of illness and death around the globe (WHO, 2014). Malaria affected 216 million people worldwide in 2016, up from 211 million in 2015. The estimated number of deaths from malaria in 2016 was 445 000, or around the same as in 2015 (446 000) (WHO, 2017). Based on the World Health Organization's 2017 projections of 219 million cases and 435,000 fatalities, over half of the world's population was thought to be at risk of contracting malaria. (WHO, 2018).

As per the latest global malaria report, there was a rise from 245 million cases in 2020 to 247 million new cases worldwide in 2021, and 619,000 malaria-related deaths (compared to 625,000 in 2020). (Werissaw Haileselassie et al., 2023; WHO, 2022). Globally, the number of cases declined steadily from 248 million to 230 million between 2010 and 2015 (Table 1). Malaria incidence has risen since 2016, with the highest yearly occurrence of thirty million more cases between 2019 and 2020, the COVID-19 pandemic's first year. There was a notably less increase in cases between 2020 and 2021, with only two million more cases (Table 1). According to WHO (2022), a further 13.4 million cases were believed to have resulted from the COVID-19 pandemic breakdowns.

Table 1.Global Estimated Malaria Cases and Deaths, 2010–2021 (WHO, 2022).

Year	Number of Cases (000)	Number of Deaths
2010	248 000	704 000
2011	242 000	660 000
2012	238 000	622 000
2013	233 000	603 000
2014	231 000	584 000
2015	230 000	577 000
2016	232 000	580 000
2017	237 000	587 000
2018	231 000	567 000
2019	232 000	568 000
2020	245 000	625 000
2021	247 000	619 000

Central and Southern America, Africa, Central, South, and Southeast Asia, as well as countries in the Pacific, are the most tropical and subtropical areas of the world where malaria is most prevalent. But underdeveloped nations are most impacted (WHO, 2017).

Malaria, which has a high rate in Southern and Central America, Africa, South, Central,and Southeast Asia, as well as countries in the Pacific, are the most tropical and subtropical areas of the world where malaria is most prevalent. But underdeveloped nations are most impacted (WHO, 2017). Malaria, which has a high death and morbidity rate, is an important public health problem in Latin America, Asia, and SSA (WHO, 2018). In SSA, where the annual infection and death rates are 191 million and 395,000, respectively, a child under the age of five dies from it every two minutes (WHO, 2019a). The African Region recorded 91% of the global malaria fatalities and 200 million cases, or 92% of malaria cases in 2017. The worldwide burden of malaria is still disproportionately high in this region (WHO, 2017). Approximately 90% of the world's death burden is in SSA (WHO, 2015a). The COVID-19 pandemic caused service interruptions between 2019 and 2020, the World Malaria Report 2022 states that malaria cases in the African Region increased from 218 million to 232 million, and malaria fatalities decreased from 544 000 to 599 000. (Table 2). While the number of patients rose to 234 million in 2021, the number of deaths fell to 593 000 (Table 2). Approximately 234 million cases (95%) and 593,000 fatalities (96%) from malaria are expected in SSA in 2021 (WHO, 2022).

Table 2. Estimated Malaria Cases and Deaths in the African Region, 2017–2021 (WHO, 2022).

Year	Number of Cases (000)	Number of Deaths
2017	218 000	555 000
2018	215 000	541 000
2019	218 000	544 000
2020	232 000	599 000
2021	234 000	593 000

Malaria continues to be a serious public health issue and is one of the leading causes of hospitalization and mortality in Ethiopia (Argawi *et al*., 2014; FMO, 2014). Among the 109 million people in the country, the WHO reported that there were 2.4 million cases in 2018 (WHO, 2019). *P. falciparum* is responsible for the majority (~60%) of the disease burden in the country as a whole, followed by *P. vivax* (~40%) (Hiwot S et al., 2018). Less than one million cases of malaria were reported in 2019, compared to 5.2 million in 2015 (Werissaw Haileselassie et al., 2023). 1.7% of malaria cases globally are still reported from Ethiopia. There were 8 041 deaths and 2 783 816 cases in 2021 in the country (Werissaw Haileselassie et al., 2023; WHO, 2022).

2.2. Cause and Transmission of Malaria

Malaria is a parasitic protozoan illness that is spread by vectors. Currently, it is found in more than 80 countries (Al-Awadhi *et al*., 2021; Sato, 2021). There are more than 200 Several Plasmodium species that infect different vertebrate species (Sato, 2021). Four species— *P. vivax, P. falciparum, P. malariae,* and *P. ovale* —are regarded as genuine parasites of humans since they virtually exclusively use humans as a natural intermediate host (CDC, 2017; Sato, 2021). It is becoming more widely acknowledged that P. knowlesi, the fifth species, is the main cause of malaria in humans (Sato et al., 2019). According to the WHO, More than 90% of malaria infections and fatalities globally in 2017 were caused by *P. falciparum* (WHO, 2018). *P. falciparum* is extremely common in most regions where malaria is endemic, particularly in Southeast Asia and Sub-Saharan Africa (SSA), where it was responsible for 62.8% and 99.7% of malaria cases, respectively (WHO, 2018).

According to Cooke *et al*. (2015), female Anopheles mosquitoes that bite mostly between dusk and dawn transmit malaria. Other somewhat uncommon transmission methods include sharing infected needles and congenital transmission (Caroline *et al*., 2013), blood transfusion (Owusu *et al*., 2010), and organ transplantation (Owusu *et al*., 2010).

The parasite, the environment, the human host, and the vector all have an impact on how intensely the infection spreads (WHO, 2014). Environmental changes that impact these vectors' density and capacity to spread the disease have a significant impact on the intensity of transmission. The duration of larval development, parasite development, and mosquito survival are all impacted by temperature. Both the Plasmodium parasites and mosquito larvae develop more quickly in warm climates (Paaijmans et al., 2009).

The vector prefers to attack people over other animals, which, in areas where mosquito lifespans are higher, enhances transmission (giving the parasite enough time to complete its development within the insect). The high percentage (90%) of malaria mortality in Africa is due to these traits, which are present in African vector species (WHO, 2014).

2.3 The Plasmodium Parasite and Vectors

Malaria is a parasitic infection caused by Plasmodium protozoan parasites, which are spread by female Anopheles mosquitos. People can contract malaria from five different kinds of

parasitic malaria: *P. vivax, P. falciparum, P. knowlesi, P. ovale and P. malariae* (Sato, 2021). *P. falciparum poses the greatest risk* and the main source of illness and death among human malaria parasites, mostly in Africa (WHO, 2020a). When the circulation is exposed to Plasmodium parasites and infiltrate red blood cells, symptoms of malaria start to manifest. Within a red blood cell, the parasite consumes hemoglobin. Hemoglobin digestion liberates large quantities of heme, which is poisonous to the parasite. Free heme is detoxified by the parasite by its crystallization into inert hemozoin that remains in the parasite and offers a docking surface for continuously supplied heme (Fitzroy *et al*., 2017).

2.4. Malaria Management and Control

According to the WHO's recent annual worldwide malaria report, 229 ,000,000 cases of malaria were anticipated to have occurred in 2019 in eighty seven countries where malaria is widespread, there has been a 9 million case drop since 2000. It was, however, more than the projected 218 million cases of malaria mentioned in the malaria Global Technical Strategy, 2016–2030 (WHO, 2020). This is redirected in the worldwide case incidence of malaria (number of cases per 1000 at-risk individuals) dropped into fifty eight in 2015 and fifty seven in 2019 compared to eighty in 2000.

Integrated vector management, early diagnosis, and efficient treatment implementation are substantially to blame for the decline in malaria death and incidence rates (Berhane *et al*., 2011). Particularly in regions with low levels of socioeconomic development, where the requisite health infrastructure is lacking, it is vital to incorporate more extensive preventive, early detection, and treatment methods for infections (Mouatcho *et al*., 2013).Global malaria incidence and mortality rates decreased by more than 50% between 2000 and 2015 (WHO, 2016). Gains in malaria control have been largely attributable to the adoption of important intervention strategies, such as vector control, early access to medication, early diagnosis, and effective treatment (Sauboin *et al*., 2019). Indoor residual spraying (IRS) and LLINs are two frequently used malaria vector control methods in SSA (WHO, 2019a).

One of the main interventions for malaria control is case management, encompassing ACT testing and treatment (WHO, 2018). In endemic areas with limited access to these measures, the drop in malaria has been slower or has stagnated (WHO, 2019). In terms of malaria prevention, the first two decades of the new millennium are considered a golden period (WHO, 2020).

In Ethiopia, there has been a notable decline in malaria morbidity and mortality over the past 20 years, in part as a result of international intervention funding (FMO, 2017), particularly following the implementation of the Health Sector Transformation Plan (HSTP) I (2015–2020), the country's national health strategic plan. In accordance with the WHO's Global Technical Strategy, Ethiopia recently established the objective of completely eradicating malaria nationally by 2030 (FMOH, 2017). However, a variety of issues, including the growth of insecticides and drug resistance, are preventing the country from achieving its goal of eradicating malaria by 2030 (Elifaged Hailemeskel et al., 2019; Hancock *et al*., 2020). Resistance monitoring has therefore drawn a lot of attention in an effort to preserve the recent advancements made.

2.5 Anti-Malarial Drugs

Quinine was initially successfully used to cure malaria, making it widely recognized as the most important medical discovery of the seventeenth century (Greenwood, 1992). This was also the first time a chemical substance has been successfully used to treat an infectious disease. The powerful and reasonably priced 4-aminoquinoline drugs chloroquine (CQ), amodiaquine, piperaquine, mefloquine, and primaquine were all developed by systematic modification of quinine. Based on less closely related ring structures, the antimalarial drugs halofantrine and lumefantrine have also been created (Fitch, 2004). Although quinine is no longer as effective against *P. falciparum* infections in much of the world due to widespread resistance, it is still effective against *P. ovale, P. malariae,* and *P. vivax* infections (Asih and Syafruddin, 2021).

2.5.1. Chloroquine (CQ) and Mechanism of Action

CQ was introduced in the early 1940s; however, *By the early 1960s, parasites resistant to P. falciparum had emerged* (Eyles *et al*., 1963). Because of widespread resistance, the efficacy of chloroquine, which was previously the preferred medication in the battle against *P. falciparum* is now severely constrained (Singh *et al*., 2016). It is common practice to treat malaria using quinoline compounds like chloroquine, but the parasite that causes the disease is quickly developing resistance to chloroquine and other quinoline chemicals are frequently used to treat malaria; however, the parasite is quickly developing resistance to these medications (Milligan *et al*., 2020). Failure of CQ therapy for *P. falciparum* and *P. vivax* was reported in Ethiopia in 1996 (Tulu *et al*., 1996). *P. falciparum* that is resistant to CQ is widely

used, SP replaced CQ as the recommended course of therapy when the country switched to it in 1998, In 2004, AL took its position as the therapy for uncomplicated falciparum malaria (Lemu Golassa et al., 2015; Elifaged Hailemeskel et al., 2019; Jifar Hassen et al ., 2022).

Based on previous studies, various hypotheses have been forwarded on the mechanism of quinoline-family drugs in damaging the parasites: i) through the hemozoin crystals' developing quinoline cap, thereby delaying the crystal surface's heme deposition (Fitzroy *et al*., 2017); and ii) complexing in the digestive vacuole lumen with free heme (Egan *et al*., 1997), however, in terms of preventing crystal growth, this process ought to be secondary. (Olafson *et al*., 2017). According to these hypotheses, the parasite suffers harm from heme that is liberated from hemoglobin but is unable to solidify. (Ch'Ng *et al*., 2011).

This weak base, called CQ, travels quickly across cell membranes and builds up in the digesting vacuole's acidic environment where it becomes protonated. (Henry *et al*., 2008). The process of haem detoxifying in the digestive vacuole is interfered with by CQ when the parasite breaks down hemoglobin. The parasite dies as a result of these haem complexes. (Henry *et al*., 2008). CQ chemically interferes with intraparasitic heme detoxification, blocking the dimerization process that culminates in the synthesis of hemozoin (pigment of malarial pigment) (Hoffman *et al*., 2011). CQ is moved into the parasite's feeding vacuole (FV) and locked there in its acidic state. The parasite Plasmodium is then eliminated by preventing the development of hemozoin (Figure 1).

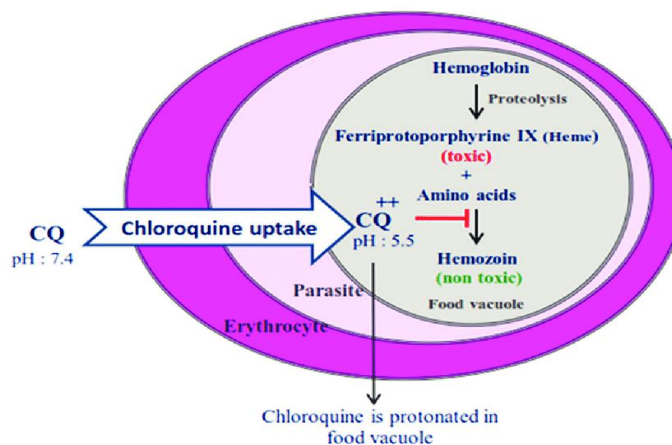


Figure 2: Antimalarial Action of Chloroquine adapted from (Parhizgar and Tahghighi, 2017).

2.5.2. Resistance to Chloroquine

Single nucleotide polymorphism in the malaria parasite that kills people the most, *P. falciparum pfmdr1-86*, located in chromosome 5 (Wurtz *et al.,* 2012), and the *pfcrt-76*, on chromosome 7, adjust the degree of resistance to chloroquine (Sidhu *et al*., 2006). Numerous investigations have shown an excellent association between the *pfcrt-T76* and *pfmdr1-Y86* mutant alleles, which have a higher level of chloroquine resistance (Kublin *et al*., 2003;Seleshi Kebede et al., 2014; Ndam *et al*., 2017). The *pfmdr1-Y86* mutation is associated with high-level CQ resistance when combined with the *pfcrt-T76* genotype (Khalil *et al*., 2005). The following lists the intracellular targets of the main antimalarial drugs and drug resistance factors (Figure 2).

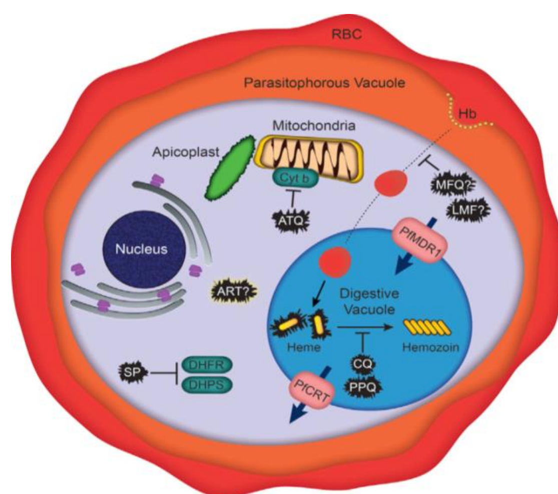


Figure 2: Intracellular Targets of Principal Antimalarial Classes and Factors Contributing to Drug Resistance.
Artemisinin (**ART**); atovaquone (**ATQ**); chloroquine(**CQ**); cytochrome b (**Cyt b**); dihydrofolate reductase (**DHFR**); dihydropteroate synthase (**DHPS**); haemoglobin (**Hb**); lumefantrine (**LMF**); mefloquine (**MFQ**); P. falciparum chloroquine resistance transporter (**PfCRT**); P. falciparum multidrug resistance-1 (**PfMDR1**); piperaquine (**PPQ**); red blood cell (**RBC**); sulfadoxine-pyrimethamine (**SP**). Adapted from (Ross and Fidock, 2019).

2.5.3. Artemisinin Combination Therapies (ACTs)

An ACT combines a derivative of artemisinin with a companion drug that is not an artemisinin. Artemisinin is crucial for lowering parasite biomass throughout the initial 3 days of treatment, even though both medicines are required for ACT to function. Any residual parasites are subsequently helped to disappear (cure) by the partner drug (WHO, 2018a). In 2006, the WHO recommended ACTs as a first-line therapy for *P. falciparum* malaria used worldwide (WHO, 2015c). According to the WHO updated Guidelines for Malaria published on February 16, 2021, the following six ACTs are recommended: artesunate + pyronaridine; artesunate + amodiaquine; artemether + lumefantrine; dihydroartemisinin + piperaquine;

artesunate + sulfadoxine-pyrimethamine (SP); and artesunate + mefloquine; are advised for the treatment of adults and children suffering from uncomplicated *P. falciparum* malaria (with the exception of first-trimester pregnant women) (WHO, 2021). Consequently, the two drugs work together to produce efficient clinical and parasitological treatments and are believed to guard against the emergence of resistance in one another. If a parasite develops mutation-associated resistance while undergoing treatment, the additional partner drug will eliminate this resistant strain (Nosten and White, 2007).

2.5.4. Artemisinin - Mechanism of Action

Artemisinin known by the name "qinghaosu,", is a drug that is semisynthetic and is made from Artemisia annua leaves (sweet wormwood). Nowadays, the mainstay of antimalarial combination treatment is artemisinin derivatives. Artemisinin derivatives do not kill mature *P. falciparum* gametocytes, but they do indirectly inhibit gametocyte growth by killing immature gametocytes and asexual erythrocytic parasites before gametocytogenesis actually occurs (Abdul-Ghani and Beier, 2014; Coertzen *et al*., 2018). Artemisinin derivatives are antimalarial drugs that work against *P. falciparum* and *P. vivax* in all of their asexual phases, both those that are resistant to and susceptible to chloroquine. They also actively combat the sexual forms of *P. falciparum* (gametocytes), which has crucial implications for malaria transmission reduction (Djimdé and Lefèvre, 2009). Artemether, Dihydroartemisinin, and artesunate are frequently used derivatives of artemisinin (WHO, 2010b).

Furthermore, Artemisinins work well not just against *P. falciparum* strains that are multi-resistant, but they also possess wide phase specificity against the life cycle of Plasmodium, involving activity during both the sexual gametocyte and asexual blood phases, potentially slowing the spread of disease in areas of low-transmission (Abdul-Ghani and Beier, 2014).

As for how artemisinin works, there are a number of hypotheses for how to artemisinin exerts its action: suppression of ATPase that transports calcium across the sarco-/endoplasmic reticulum membrane, disruptions to the detoxification of haem, disruption of the function of the mitochondria, and triggering the alkylation of tumor proteins under translation control (Premji, 2009). Artemisinin is thought to have a two-step mechanism of action. This endoperoxide is cleaved by intraaparasitic heme-iron, activating artemisinin in the process. An intermediate free radical produced as a result of this reaction may perhaps alkylating the parasite to death and harm a few important malarial proteins (Meshnick, 1998).

2.5.5. Artemether-lumefantrine (AL) - Mechanism of Action

In 2009, the Food and Drug Administration (FDA) approved AL for the treatment of kids, adults, and newborns with uncomplicated *P. falciparum* malaria (Stover *et al.*, 2012; Thwing *et al.*, 2009). A blood schizonticide called lumefantrine is effective against Plasmodium falciparum's erythrocytic stages. Lumifantrine with artemether administration is believed to have synergistic antimalarial clearance effects. Artemether acts quickly and is quickly eliminated from the body. Thus, it is believed to quickly relieve symptoms by lowering the parasite load of malaria. (Thwing *et al.*, 2009). Lumefantrine is thought to eliminate remaining parasites because of its substantially longer half-life. Artemether and lumefantrine's pharmacokinetics allow them to work together to offer quick symptom alleviation and a high percentage of parasite cure. Artemether starts working quickly, reducing the severity of the symptoms and rapidly lowering the biomass of parasites (Djimdé and Lefèvre, 2009).

Unknown is the precise mechanism through which lumefantrine works to prevent malaria. However, the information that is now available indicates lumefantrine's inhibition of the production of nucleic acids and proteins and prevents the development of -hematin by establishing a hemin-containing compound (Thwing *et al.*, 2009). It is utilized in individuals suffering from uncomplicated *P. falciparum* malaria in conjunction with artemisinin derivatives. AL is the only drug that may be used in conjunction with lumiefantrine (Abdulhakim Abamecha et al., 2020).The medication quickly eliminates parasites and reduces the number of gametocyte carriers (Figure 3). This ACT is a cornerstone of ACT in Africa and is very effective against *P. falciparum* (WHO, 2010c).

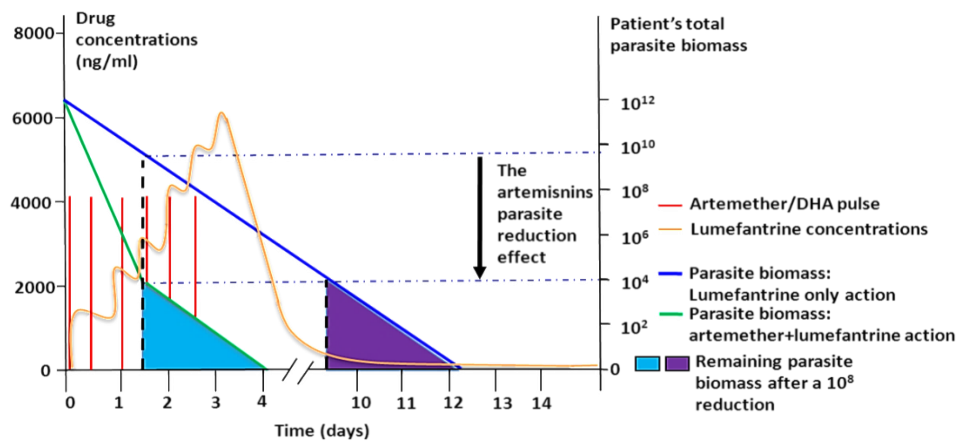


Figure 3. The ACT Treatment's Artemether-Lumefantrine Principle.
When treated to lumefantrine in monotherapy, the whole biomass of parasites is represented by the huge triangle under the blue line. The artemisinin derivative in ACT quickly lowers the biomass of parasites (green line), and the turquoise triangle represents the tiny percentage of remaining parasites that come into contact with lumefantrine. Lumefantrine is much more concentrated in these parasites (area under the orange curve) than the biomass of the same parasite in monotherapy that was exposed to lumefantrine (purple triangle). Adapted from White, 1997.

2.6. Resistance to Artemisinin Combination Therapies (ACTs)

Partner drugs resistance, artemisinin resistance, or both can be contributors to resistance to ACT (Duru *et al*., 2016; Menard and Dondorp, 2017). An indication of growing resistance is a slowdown in the removal of parasites. The primary indicator of artemisinin resistance is delayed parasite elimination. (Indicating a decline in the ring-stage parasites' susceptibility) (Muhindo *et al*., 2014). Hence, few parasites will be cleared, leaving more parasite biomass for the partner drug to clear. This activity makes partner drug selection easier and raises the chance of treatment failure (Fairhurst and Dondorp, 2016; White, 2016). Late treatment failure might be a symptom for resistance of partner drugs, when 4-6 weeks after starting anti-malarial medication, a patient who has previously eradicated their parasites gets recurrent parasitaemia (Amaratunga *et al*., 2016; Beshir *et al*., 2021). A substantial decrease in cure rates occurs from resistance to both ACT components (Nsanzabana, 2019).

P. falciparum resistance to ACTs was reported in Thailand, Myanmar, Vietnam, and western Cambodia (Ashley *et al*., 2014; Thriemer *et al*., 2014), where the rates of ACT therapy failure are rising quickly (WHO, 2015b). In this region, decreasing the efficacy of the combination of mefloquine and artesunate (Wongsrichanalai & Meshnick, 2008) and slow parasitaemia clearance following treatment as a sign of clinical resistance to artesunate, were observed (Phyo *et al*., 2012). Moreover, recurrence of infections after treatment with DP has been reported in other studies from Cambodia, raising concerns that the spread of artemisinin

resistance has accelerated the spread of artemisinin partner drug resistance (Saunders *et al*., 2014).

ACTs are the gold standard for treating *P. falciparum* malaria in SSA, despite the fact that resistance to artemisinin has arisen in western Cambodia and neighboring areas (Dondorp *et al*., 2009). A major danger to the world's public health is artemisinin resistance, having the worst effects in the SSA, where the prevalence of disease is higher, and there are inadequate monitoring and containment systems. (Slater *et al*., 2016). Both Southeast Asia and Africa are seeing widespread artemisinin resistance (delayed clearance of *P. falciparum* with ACT treatment). Studies from Kenya, Equatorial Guinea, Madagascar, Zambia, Tanzania, Somalia, Papua New Guinea, Senegal, and Ethiopia shown remarkable efficacy with AL (>90%) (Abdulhakim Abamecha et al., 2020; Dentinger *et al*., 2021; Diallo *et al*., 2020; Ippolito *et al*., 2020; Kishoyian *et al*., 2021; Mandara *et al*., 2018; Seleshi Kebede et al., 2015; Riloha Rivas *et al*., 2021; Tavul *et al*., 2018; Warsame *et al*., 2019). A different African study revealed that AL therapy was ineffective for treating uncomplicated *P. falciparum* malaria in travelers from Mozambique and Angola (Silva-Pinto *et al*., 2021).

2.7. *pfmdr1-86* and *pfcrt 76* Genes and Mutation

2.7.1. *pfmdr1-86* Gene and Mutation

pfmdr1, alternatively referred to as *Pgh-1*, the human cancer cells' homologue of mammalian P-glycoproteins, which mediates drug resistance, was studied long before. The primary factor responsible for CQ resistance was found to be *pfcrt* (Cowman *et al*., 1991). The *pfmdr1* gene, which is located on the fifth chromosome, gives rise to a 162-kDa protein that is mostly present in the membrane of the parasite's digesting feeding vacuole (Duraisingh and Refour, 2005). *pfmdr1*, one of the transporters belonging to the superfamily of ATP-Binding Cassette (ABC), is encoded by the gene *pfmdr1*. The protein is present in the digesting vacuole's membrane and, comparatively less in the plasma membrane (Duraisingh and Cowman, 2005). It is believed that *pfmdr1* is directed towards the digestive vacuole and capable of importing compounds, including certain antimalarial drugs, into the lumen of digestive vacuole (Ferreira *et al*., 2011).

In vivo and in vitro associations have been found for five *pfmdr1* polymorphisms.: D1246Y (D = aspartic acid, Y = tyrosine), S1034C (S = serine, C = cysteine), , N1042D (N = asparagine) , Y184F (F = phenylalanine), and N86Y (Sidhu *et al*., 2005) with decreased

vulnerability to serious ACT drugs like artemisinin (Boonyalai *et al*., 2021), and lumefantrine (Chidimatembue *et al*., 2021; Fatou Kene et al., 2021).

Resistance to various anti-malarials and a sudden expansion in multidrug parasites are found to be caused by variation in mRNA expression levels, amplification, and polymorphism of the *pfmdr1* gene (Duraisingh and Refour, 2005). In *P. falciparum*, multiple transporters, including the *pfmdr1* gene, have been associated with altered sensitivity to antimalarial drugs (Petersen *et al*., 2011). The selection of specific parasite genotypic variations, such as *pfcrt K76* and *pfmdr1 N86*, has been connected to ACT treatment failures (Malmberg *et al*., 2013). Mefloquine (MQ), AQ, and CQ resistance have all been associated with the *pfmdr1* gene. SNPs located at codons 1246 (D1246Y) , 184 (Y184F), and 86 (N86Y) result in decreased susceptibility of parasites to certain drugs, such as ACT (Holmgren *et al.,* 2007; Sisowath *et al.,* 2007). For instance, when the *pfcrt T76* genotype is paired with the *pfmdr-1 Y86* mutation, high-level CQ resistance is linked to it. (Duraisingh and Cowman, 2005; Khalil *et al*., 2005).

SNPs are among the genetic mechanisms underlying the resistance to chloroquine within chromosome 5's *pfmdr1*, which codes for a multidrug resistant P-glycoprotein homologue-1 transporter found in the food vacuole of the parasite and is linked to increased drugs efflux from parasites that have developed resistance (Foote *et al*., 1990). The codons 1246, 1042, 1034, 184, and 86 are where the *pfmdr1* mutations associated with antimalarial drug resistance are found (Duraisingh *et al*., 2000). The mutation that results from asparagine replacing tyrosine at position 86 is connected to resistance to chloroquine (Duah *et al*., 2007).

2.7.2. *pfcrt 76* Gene and Mutation

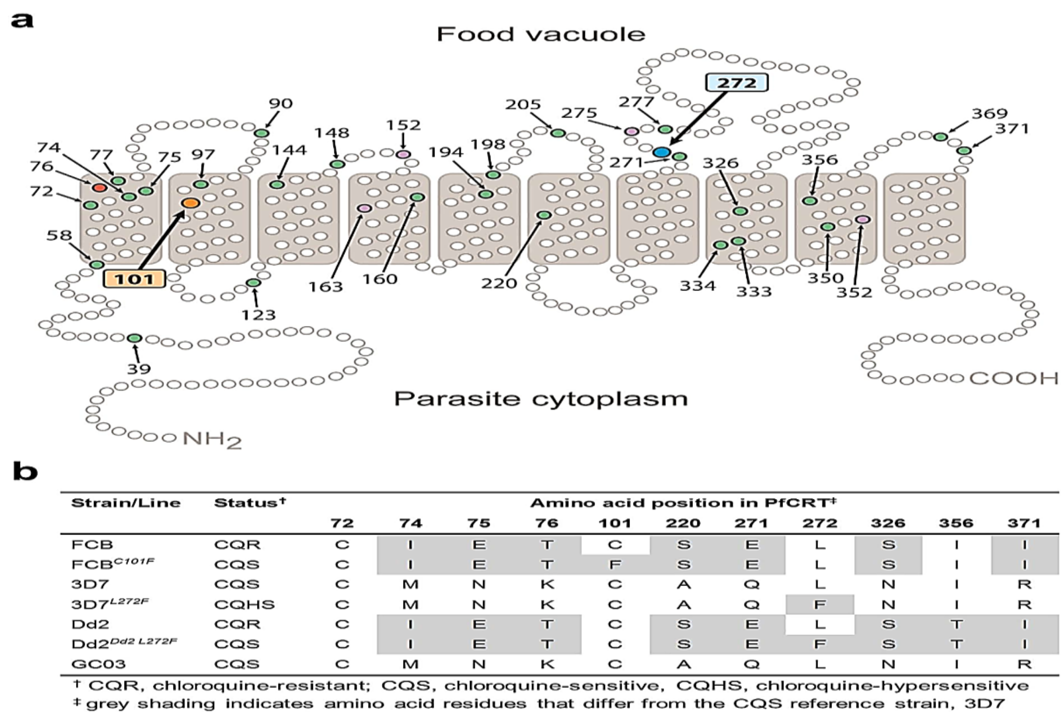
A key gene, *pfcrt*, was discovered in chromosome 7 loci, providing the ten anticipated transmembrane domains of a 45 kDa protein. This putative protein is known to localize into the membrane of the digestive vacuoles of parasites (Cooper *et al*., 2002). *pfcrt* is thought to be a critical protein involved in cell function since a pfcrt knockdown has been demonstrated to be lethal for parasite survival (Waller *et al*., 2003). Its lethality is a barrier to a precise knowledge of its endogenous substrates and natural function. *pfcrt* function has been the subject of much discussion in the pursuit of understanding. The malaria parasite's drug resistance mechanism. There has been a lot of debate on the process via which mutant *pfcrt* decreases the buildup of CQ in the DV and confers resistance (Sanchez *et al*., 2010). *pfcrt*

homologs have been discovered in plants in recent investigations, and they most likely have a role in stress reactions and glutathione homeostasis (Maughan *et al*., 2010).

Eight codon variations were found in the sequence comparison between the Dd2 and HB3 pfcrt open reading frames (Fidock *et al*., 2000). Assessment of numerous geographically distinct CQR and the most reliable molecular marker of resistance was found to be a particular codon shift at position 76, from lysine to threonine, using CQS clones (Valderramos and Fidock, 2006) (Figure 4). *pfcrt* as important factors influencing reduced in vitro sensitivity to many main antimalarial drugs. One of the variables linked to failure of CQ treatment in Africa is the *pfcrt* gene T76 and *pfmdr1* gene Y86 mutations in the, as shown by genotype-failure indices (GFI) (Dacuma *et al*., 2021).

Preliminarily, data from previous episomal expression systems and the allelic exchange technique revealed this SNP's main influence on *P.falciparum* response to CQ in vitro. (Fidock *et al*., 2000; Lakshmanan *et al*., 2005). However, this single mutation has not been able to be introduced into a chloroquine-sensitive (CQS) parasite, indicating that it could have a functional impact, but additional pfcrt mutations might offset it.

In addition to CQ, the resistance of the parasite to different antimalarial drugs can be influenced by the *Pfcrt* transporter in vitro, mainly quinoline-based, however artemisinin is also included (Lakshmanan *et al*., 2005). In the context of the ACT, resistance to artemether-lumefantrine may also be influenced by this gene. It has been noted that the pfcrt K76 is selected in re-infections after artemether-lumefantrine treatment (Otienoburu *et al*., 2016), a finding backed up by in vitro studies indicating that the K76 is linked to lower sensitivity to lumefantrine (Mwai *et al*., 2009).



Strain/Line	Status†	Amino acid position in PfCRT‡										
		72	74	75	76	101	220	271	272	326	356	371
FCB	CQR	C	I	E	T	C	S	E	L	S	I	I
FCB^{C101F}	CQS	C	I	E	T	F	S	E	L	S	I	I
3D7	CQS	C	M	N	K	C	A	Q	L	N	I	R
3D7^{L272F}	CQHS	C	M	N	K	C	A	Q	F	N	I	R
Dd2	CQR	C	I	E	T	C	S	E	L	S	T	I
Dd2$^{Dd2\ L272F}$	CQS	C	I	E	T	C	S	E	F	S	T	I
GC03	CQS	C	M	N	K	C	A	Q	L	N	I	R

† CQR, chloroquine-resistant; CQS, chloroquine-sensitive, CQHS, chloroquine-hypersensitive
‡ grey shading indicates amino acid residues that differ from the CQS reference strain, 3D7

Figure 4: Gene Mutations Associated with *Plasmodium falciparum Chloroquine Resistance Transporter (pfcrt).*

(a) A schematic of the pfcrt and the locations of previously found polymorphisms (Ecker *et al*., 2012) 8,71 from drug-pressured laboratory lines and field isolates (green and purple circles, respectively). Red shading indicates the crucial CQ resistance mutation locus (K76) and the two residues at which mutations are described in this study are shaded in orange (C101) and blue (L272). (b) in the *pfcrt* haplotypes of the previous study. Adapted from Pulcini *et al*. (2015).

The key single nucleotide polymorphism (SNP) responsible for the resistance phenotype is found at codon 76, where threonine is substituted for lysine in the sequence of the gene and the most credible molecular indicator of resistance to chloroquine among the many mutations found (Dijmde *et al*., 2001; Ibrahim *et al*., 2009). A T76 mutation in the *pfcrt* gene is linked to chloroquine resistance (Fidock *et al*., 2000), yet its degree may be modulated by an Y86 mutation of the multidrug resistance analogue (*pfmdr1*). (Sanchez and Lanzer, 2000). This mutation is linked to the digesting vacuole, the CQ action compartment, becoming more acidic (Fidock *et al*., 2000).

The *pfmdr-1* and *pfcrt* genetic markers have been linked to CQ resistance in earlier research (Lim et al., 2009). Chloroquine resistance linked to the two genes mutations: pfmdr1 at codon 86, which transforms asparagine (N86) to tyrosine (Y86), and pfcrt at codon 76, which causes

lysine (K76) to convert to threonine (T76) (Plowe *et al*., 1999). It has recently been determined that point mutations in pfcrt are the primary predictor of chloroquine resistance. The following supports this evidence: (i) In a *P. falciparum* genetic cross, these mutations segregate with CQR (Fidock *et al*., 2000; Wellems *et al*., 1990); to ascertain the molecular mechanism of CQ resistance, a genetic cross between CQR, the Dd2 clone clone and CQS, the HB3, was employed (Fidock *et al*., 2000). (ii) In field isolates modified for the laboratory from various geographically varied places, mutant *pfcrt* haplotypes reveal a significant relationship with CQR (Fidock *et al*., 2000); (iii) A greater rate of failure of CQ treatment is often linked to *pfcrt* gene point mutations (Sutherland *et al*., 2002); (iv) under CQ pressure, a recent global sweep of pfcrt haplotypes mutaion is supported by microsatellite markers (Wootton *et al*., 2002); and (v) allelic exchange data has revealed compelling evidence that a CQS clone may receive CQR from mutant *pfcrt* alleles distributed across South America, Africa, Asia, and Oceania. (Sidhu *et al*., 2002). Since then, P. falciparum populations have employed these changes as molecular indicators of chloroquine resistance (Jelinek *et al*., 2002) and are an efficient epidemiological instrument for tracking drug resistance to malaria treatment drugs (Djimde *et al*., 2001).

Simple molecular techniques, such as the very sensitive and specific PCR/RFLP, can be used to identify the existence of these mutations (Cortese and Plowe, 1999). Due to changes in these drug-resistance genes, the *pfmdr-1* and *pfcrt* genes are associated to CQ resistance in the malaria parasite (Haldar *et al*., 2018). It was previously believed that polymorphisms in the gene of pfcrt, which is found on chromosome 7, had an impact on chloroquine resistance. Transfection studies have demonstrated that the 76-Ser polymorphism is substantially linked to the resistance phenotype.

Table 3: *pfcrt76* and *pfmdr1-86* Genes Single Nucleotide Polymorphism Profile (Ngum *et al*., 2022).

Gene	Locus	Amino acid	Genotype	Alleles
pfmdr1	TGA	Asn	Wild type	*N86*
	TGT	Tyr	Mutant	*86Y*
	TGA/TGT	Asn/Tyr	Mixed	*N86Y*
pfcrt	AAA	Lys	Wild type	*K76*
	ACA	Thr	Mutant	*76T*
	AAA/ACA	Lys/Thr	Mixed	*K76T*

The monitoring of resistance to anti-malarial drug using molecular surveillance may offer valuable insights into the current state of anti-malarial drug resistance and aid in tracking its dissemination. The present study aimed to assess the prevalence of *pfcrt-76* and *pfmdr1-86* gene mutations to make an appropriate intervention in the malaria elimination campaign. The finding will offer critical information for the monitoring and early identification antimalarial drugs resistance of *P. falciparum* in the country. Therefore, it is essential to accurately measure the antimalarial drugs resistance of *P. falciparum* strain's in relation to the malaria burden in order to plan national health services, track progress toward targets, and concentrate future efforts.

3. Materials and Methods

3.1. Description of the Study Area

Based on documented variations of co-endemicity levels of *P. vivax* and *P. falciparum*, study locations were chosen (MOH, 2016). Four Districts (Fentale, Metemma, Dera and Abobo) on behalf of diverse epidemiological situations from 3 regional states (Oromia, Amhara and Gambella) of Ethiopia were included.

The Fentale District is part of the Great Rift Valley's Oromia Region's east Shewa Zone. The Arsi Zone borders Fentale on the southeast, Boset on the southwest, the Amhara Region on the northwest, and the Afar Region on the northeast. The altitudes in most parts of this district range from 900 to 1000 masl, except for the highest point, Mount Fentale (2400 meters). The administrative center of Fentale, Metehara, is located 188km east of Addis Ababa and has a latitude and longitude of 08°54′N 39°55′E at an elevation of 947 masl. The area receives 500–650mm of annual average rainfall, and the ranges of average yearly temperatures from 220C to 290C. The estimated total population of districts is 87,424, of whom 41,437 are men and 45,987 are women, within a total area of 1,170 km2. The district's water bodies include the Awash and Germama rivers and Lake Basaka (https://en.wikipedia.org/wiki/Fentale). The sugar factory found in the district and its location along the Ethio-Djibouti road play a role in the dissemination of malaria parasites to the highland areas where the migrants originated. The district has ongoing malaria transmission, with an estimated prevalence of 19.6% in the district, of which 82.4% infection was with *P. vivax* and 17.6% with *P. falciparum* (M Sleshi et al., 2012). In the district, five health centers are found.

One of the west Gondar Zone districts of Ethiopia's Amhara Region, Metemma is bordered on the south by Qwara, the north by west Armachiho, the northeast by Tach Armachiho, the east by Chilga, and the southeast by Takusa. It is situated in the lowlands on the western border with Sudan. The administrative center is Shehedi, and Metemma is the second-largest town in the district. Metemma is situated 897 km north of Addis Ababa and 197 km from Gondar, and it has a latitude and longitude of 12°58′N 36°12′E. The district has 123,235 inhabitants overall and a total area of 6,969.97 km2 (Wossenseged Lemma, 2020). The district is in a lowland area, and its altitudes range from 550 to 1600 meters above sea level. The Metema district has a hot, semi-arid climate, with 20°C to 37°C being the range for the yearly average temperature. The annual precipitation totals vary from 450 to 650 mm.

According to Getachew *et al*. (2013), the district had a Plasmodium infection rate of 17% (9486/55,833), of which 90.7% (8602/9486) infections were with *P. falciparum* and 9% (852/9486) with *P. vivax*. In the district, several mechanized farms are presented that attract a huge number of seasonal migrant labour workers from the highlands of the Amhara and Tigray regions (Chritz, 2017). In the district, five health centers are found.

One of the districts of Ethiopia's south Gondar Zone, Amhara Region, is called Dera. With a latitude and longitude of 11°45′N and 37°30′E, the capital city of Anbesame is situated 12km from the main Addis Ababa-Gondar route and 30km from Bahir Dar. The Abay River, which divides Dera from the west Gojjam Zone, is its southern boundary. Lake Tana, Fogera, the northeastern and eastern Estie, and the western and eastern Estie are its western and eastern borders, respectively. The district comprises a total of 220762 people within a total area of 1,525.24 km2. The altitude of the district ranges from 1,500 m to 2,600 m, while the annual average rainfall is 1,250 mm, and most of the area is covered with marsh areas and extensive water bodies with an average annual temperature of 17°C. In the district, frequent malaria outbreaks were reported (Amir Alelign *et al*., 2018; Mulugojjam Andualem , 2014). Plasmodium infection in the district was 12% (30/251), of which 83.3% (25/30) were infected with *P. vivax* and 16.7% (5/30) with *P. falciparum* (Awoke Minwuyelet et al., 2020). In the district, eleven health centers are located.

The Gambella Region is located in the lowlands with high levels of malaria transmission. High levels of human migration, both from and to Ethiopia for seasonal labour, and from bordering South Sudan, facilitate malaria transmission and the dissemination of new parasite variants to malaria-free areas. Abobo is one of the districts in Ethiopia's Anuak Zone, which is part of the Gambella Region. Abobo is surrounded by the Mezhenger Zone in the southeast, Gog in the south, Jor in the southwest, Itang Special District in the northwest, Gambella Zuria in the north, and the Oromia Region in the northeast. The administrative town of the district, Abobo, is located 47 km south-east of Gambella Town and at a coordinate of 070 50′ 59″ N 34032′ 59″ E and at 540 masl. The woreda has a total population of 15,741, of whom 8,184 are men and 7,557 are women, with an area of 3,116.17 km^2. Previous reports showed that there is high malaria prevalence (>18%) in the district (Shitaye *et al*., 2018), and antimalarial resistance development was also reported (Elifaged Hailemeskel et al., 2019). The district is characterized by a *P. falciparum*-dominated area (97% of all Plasmodium infections are *P. falciparum*)

(Tsegaye *et al.,* 2014) with perennial transmission. In the district, four health centers are found.

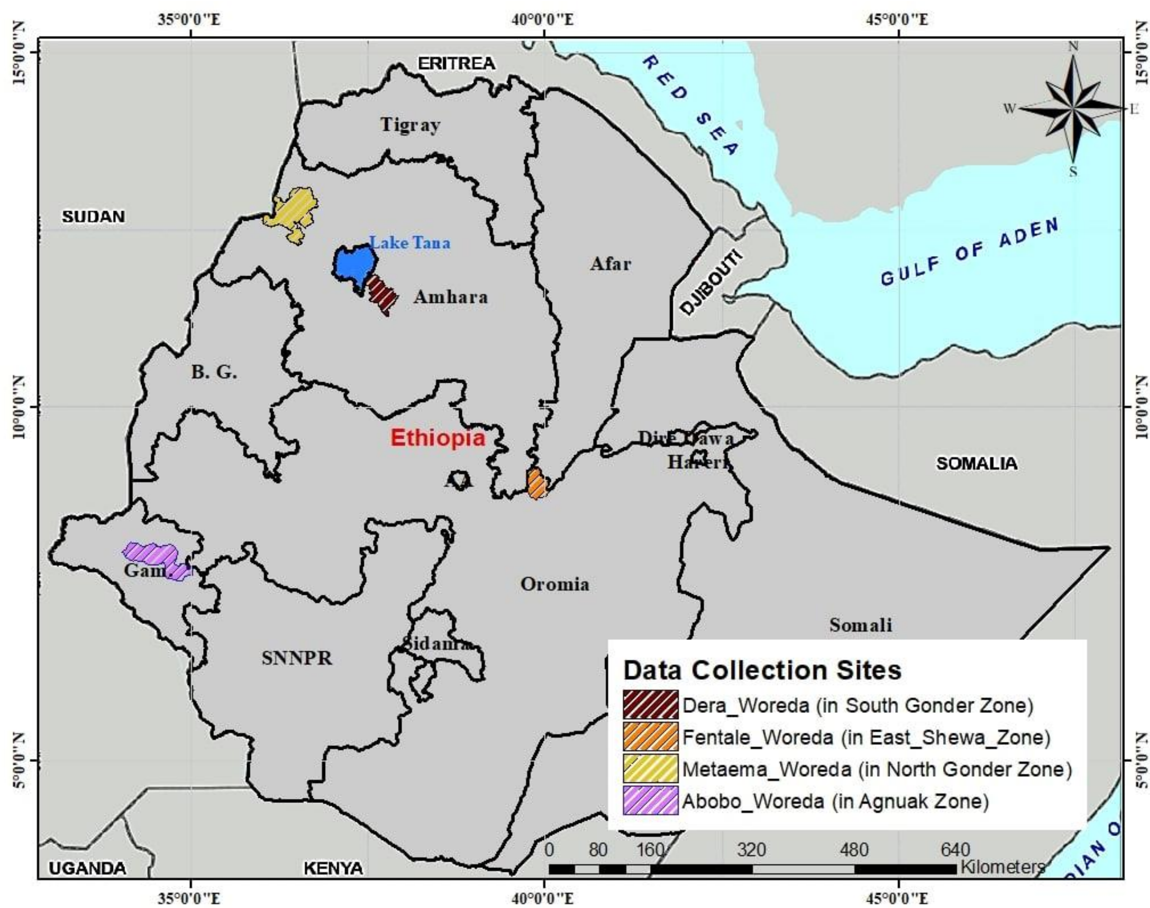


Figure- 5: Location of the Study Area

(Source- Author Design Using GIS Version 9.3 and CSA Data, 2017)

3.2. Study Design

A prospective cross-sectional study was conducted to determine the prevalence of *pfcrt76* and *pfmdr1-86* gene mutations in clinical isolates of *P. falciparum* obtained from symptomatic patients from the study sites (Fentale, Dera, Metemma, and Abobo districts) who visited the chosen health centers during the study period (from July 2021 to October 2021).

3.3.Sample Size Determination and Sampling Procedure

Using a formula appropriate to assess the difference between two population proportions and the assumptions of a 95% confidence level and 80% power, the estimated sample size for the current cross-sectional study was determined. Thus, the total calculated number of participants was as follows:

$$n = (Z_{\alpha/2}+Z_{\beta})^2 * (p_1 (1-p_1)+p_2(1-p_2)) / (p_1-p_2)^2$$

Where

- ✓ α = level of significance (Type I error)=0.05
- ✓ β = power of the study (Type II error)=0.2
- ✓ The normal distribution's critical value at $\alpha/2$ with a 95% confidence level, 1.96 is the crucial value ($Z\alpha/2$).
- ✓ The normal distribution's critical value at β for with a power of 80%, the critical value (Z_{β}) is 0.842.
- ✓ p1 is the expected proportion of *P.falciparum* dominant areas, expressed as decimal (13.5% is 0.135) (Elifaged Hailemeskel et al., 2019) and
- ✓ p2 is expected proportion of high *P.vivax* co-endemic areas, expressed as decimal (25.5% is 0.255) (Elifaged Hailemeskel et al., 2019; Seleshi Kebede et al.,2014).

Therefor $$n = \frac{(1.96+0.842)^2\ (0.135(1-0.135)+0.255(1-0.255)}{(0.135-0.255)^2} = \mathbf{167.25} \approx \mathbf{168}$$

The samples (n = 168) were allocated to the *P. falciparum* dominant area and the high *P. vivax* co-endemic area based on the *pfmdr1* mutation proportion (Lo et al., 2017) (see Annex 1.1). The samples (n = 168) were collected from Abobo (n= 83) and Dera (n= 85). For the strength of the study, an additional 90 samples were added from Metemma (n = 45) and Fentale (n = 45), representing *P. falciparum* dominant area as Abobo and high plasmodium vivax co-endemic area as Dera, respectively. A total of 250/258 and 257/258 samples were analyzed for *pfcrt76* and *pfmdr1-86* gene mutations respectively by using the PCR-RFLP method.

The districts and the health centers in the study sites were selected purposefully (considering patient flow and logistics), but patients were selected randomly with systematic random sampling.

3.4.Study Population

All patients who have given written informed consent and are residents of the study districts attending the selected health centers have a *P. falciparum* mono-species infection detected by microscopy.

3.5. Inclusion and Exclusion Criteria

3.5.1. Inclusion Criteria

All the study districts' inhabitant patients attending the selected health centers with uncomplicated *P. falciparum* mono-species infection detected by microscopy were included.

3.5.2. Exclusion Criteria

All the study inhabitant patients attending the selected health centers other than those of uncomplicated *P. falciparum* mono-infection were excluded.

3.6. Sample Collection and Management

The samples were taken from willing subjects who were diagnosed as positive for *P. falciparum* by light microscopy at the selected health centers. Informed written and signed

consent and/or assent were gained from all subjects and/or the guardians of the children below 18 years, respectively, before sample collection (see Annex 1.2). Basic demographic data were taken using a pre-tested questionnaire adopted from the WHO template (WHO, 2020).

3.7. Blood Sample Collection

Samples of finger prick blood were drawn and placed in EDTA microtainer containers. Blood in microtainer tubes was used to prepare thin and thick smears for confirmation and quantification of parasite densities by expert microscopists at the malaria research center of Armauer Hansen Research Institute (AHRI) at Adama and to prepare three dried blood spots, each of 20µL on Whatmann 3MM filter papers (Whatman, Maidstone, UK) (Fitsum Girma et al., 2017) for parasite deoxyribonucleic acid (DNA) extraction for *pfmd1-86* and *pfcrt76* gene mutation analysis. The dried blood spots (DBS) samples were air dried and stored in a freezer in zip-locked plastic bags containing self-indicating silica gel desiccant beads (Geejay Chemicals Ltd.), transported to the laboratory at AHRI at ambient temperature, and stored in a freezer at -20 °C until being processed. All survey microscopy slides and DBSs are on filter paper, labeled only with the survey ID. Until the survey findings are processed and the report is finished, DBS was kept in zip-locked plastic bags in case it was required to address data discrepancies or for additional DNA material (WHO, 2020).

3.8. Sample Processing and PCR-RFLP Analysis

3.8.1. Microscopic Examination

Finger prick blood was used to create both thin and thick blood films of a single subject on a single slide. Once the thick and thin films had fully dried, just the thin layer was fixed with 100% pure methanol for 30 seconds and dried in a horizontal postion all the way through before staining. Both the thick and thin smears were dyed with a 10% Giemsa solution for 30 minutes after they had completely dried. The stain was washed from the slide by gentle dipping into clean water in the jar. Blood film microscopy analysis was carried out.

Following the guidelines of the Basic Malaria Microscopy Standard Protocol, the stained thick blood films were evaluated (Herman *et al*., 2019) with a light microscope at x10 ocular

and x100 objective lenses with the addition of immersion oil to check for malaria parasites, and parasite speciation was done. *P. falciparum* was identified by the presence of crescent-shaped gametocytes and a high proportion of infected red blood cells when looking the smear under the microscope. Some RBCs was doubly parasitized with two ring trophozoites stage of *P. falciparum* in one cell. Asexual stage densities were simultaneously assessed by counting parasites within 1,000 leukocytes in the thick smear. Parasite counts were converted into numbers of parasites per microliter by assuming a standard count of 8,000 leukocytes/µl of blood (Herman *et al*., 2019). The following formula was used to calculate the parasite's density (Desalegn Nega et al., 2015).

Parasite density/µl = No. of asexual parasites counted × 8 × 10^3 WBCs/µl / 200WBCs

Parasitaemia was classified as low (<1000 parasites/µl of blood), moderate (1000–9999 parasites/µl of blood), and high (≥10,000 parasites/µl of blood) (Jifar Hassen et al ., 2022).

3.8.2. DNA Extraction

Genomic DNA from a punch of dried blood spots (DBS) with a diameter of 6 mm on a Kingfisher Flex automated extractor was extracted by using MagMAX™ DNA Multi-Sample Kit protocol (https://www.thermofisher.com/order/catalog/product/4413020). The detailed SOP can be found in the annex section (annex-1.3) of this thesis. The eluted DNA was either utilized right away or kept for subsequent use at -30 to -15°C.

3.8.3. Detection and Confirmation of *P. falciparum* using qPCR Assay

Rougemont *et al.* (2004) described the use of a real-time PCR assay for *P. falciparum* identification and confirmation (see Annex 1.4). This is a dual duplex test that uses species-specific TaqMan probes to identify *P. vivax* and *P. falciparum* in the same reaction. A primer and probe sequence specific to small subunits of rRNA (18S) block 9 regions, which was designed by Rougemont *et al*. (2004), was used. A forward primer, Plasmo 1 (5′-GTTAAGGGAGTGAAGACGA TCAGA-3′), and a reverse primer, Plasmo 2 (5′-AACCCAAAGACTTTGATTTCTCATAA-3′), were employed to amplify two or four plasmodial 18S genes, spanning a 157–165 bp segment. To distinguish between the species, two probes unique to each were used: *P. falciparum* labeled with 5′FAM and 3′TAMRA

(Falcprobe: 5′-FAM-AGCAATCTAA AAGTCACCTC GAAAGATGAC T-TAMRA-3′), and *P. vivax* labeled with 5′VIC (6-carboxyfluorescein) and 3′TAMRA (Vivprobe: 5′-VIC-AGCAATCTAA GAATAAACTC CGAAGAGAAA ATTCT-TAMRA-3′) (Perandin *et al*., 2004). To enable sensitivity evaluation, plasmids containing the block 9 region of the 18S gene were created for each species or used as positive controls.

Real-time PCR with species specificity for Plasmodium was designed as a duplex PCR for the identification of the *P. vivax* and *P. falciparum*. Detection and confirmation of *P. falciparum* species were performed in single reactions. Briefly, Plasmo1 and Plasmo2 primers were combined with the two TaqMan probes that corresponded to *P. vivax* and *P. falciparum*. In this PCR test, which was finished in a final volume of 25 ul, the Plasmo 1 and Plasmo 2 primers, 200 nM each, 80 nM Falcprobe and 80 nM Vivprobe, and 12.5 ul of TaqMan Universal Master Mix (Applied Biosystems) were used. Applied Biosystems' ABI Prism 7700 was used for the amplification. There was an inhibition control (spiked specimen) and a negative extraction control for every sample. For the PCR, the following conditions were applied: an initial step at 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 1 min. By determining the threshold cycle number (Ct) at which normalized reporter dye emission rose above background noise, the sample was deemed positive. The sample was deemed negative if the fluorescent signal did not rise within 40 cycles (Ct 40).

All duplex PCR reactions were accomplished in duplex/triplicate in order to evaluate repeatability the same condtions were applied for both the amplification processes and the previously mentioned interpretation of a positive vs negative result. Plasmodium species samples with CT values less than 35 were utilized for the further stages of sample preparation. Samples that did not test positive for malaria (CT value >35) were deemed ineligible (Lucchi *et al*., 2013).

3.8.4. Amplification of *pfmdr1*-86 and *pfcrt 76*

Using outer (N1) and inner primer pairs (N2), *pfmdr1-86* and *pfcrt 76* genes were further amplified for nPCR in samples that were verified by 18S RNA rPCR to be *P. falciparum* mono-species infections. Successively amplifed DNA samples were 257/258 and 250/258 for the *pfmdr1-86* and *pfcrt76* codons, respectively. PCR was tailored and adjusted based on the

research conducted by Zahon *et al.* (2021). Briefly, Using pairs of forward and reverse primers surrounding codons 86 and 76 of the relevant genes, the *pfmdr1-86* and *pfcrt 76* genes of the parasite DNA were amplified in a two-round PCR. The initial amplifications' byproducts served as the second amplification's substrates. Instead of using parasite DNA for the PCR conditions, nuclease-free water was used as the negative control. A recognized positive control of 3D7 for wild-type (pfcrt-76K allele and pfmdr1N86) codons for both *pfcrt-76* and *pfmdr1-86* and pfcrt76T and pfmdr1-86Y, two known positive controls of Dd2 for mutants and a negative control of nuclease free water were incorporated into each reaction (thanks to the Armauer Hansen Research Institute).

3.8.4.1.Amplifcation of the *pfcrt-76* Gene

Briefly, the primers N1FP-Pfcrt-76 (5'CCGTTAATAATAAATACACGCAG-3') and N1RP-*pfcrt-76* (5'GGATGTTACAAAACTATAGTTACC-3') were used in the first round of the nested PCR for the gene *pfcrt76*. In primary PCR, a reaction volume of 25.0 µl was utilized: 11.3 µl of nuclease-free water, 0.625 µl of each 0.25 M forward and reverse primer, 5 µl of 1X polymerase buffer, 2 µl of 25 mM MgCl2, 0.25 µl l of 0.25 Mm dNTPs, 0.2 µl of 1 u/ µl Taq Polymerase, and 3.0 µl of DNA template. In a thermal cycler (VWR) (Schmidt, Germany), PCR was carried out. Pre-denaturation was carried out at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 sec, 56 °C for 45 sec, and 72 °C for 1 min, and finally final elongation was carried out at 72 °C for 10 min. The initial PCR was then followed by a 2^0 nested PCR. For the second round of the nested PCR, the forward and reverse primers N2FP-Pfcrt-76 (5'-TGTGCTCATGTGTTTAAACTT-3') and N2RP-Pfcrt-76 (5'-CAAAACTATAGTTACCAATTTTG-3') were used in turn. 0.625 µl l of each 0.25 M forward and reverse primer, 5 µl of 1X polymerase buffer, 2 µl of 25 mM MgCl2, 0.25 µl of 0.25 Mm dNTPs, 0.2 µl of 1 u/ µl Taq Polymerase, and 2.0 µl of primer PCR product were used in the amplification reaction volume of 25 µl. This is how the nested PCR was carried out: Denaturation at 95 °C for five minutes at a hot start, then 30 cycles of annealing at 48 °C for 45 seconds, extension at 72 °C for one minute, and final extension at 72 °C for ten minutes.

3.8.4.2. Amplifcation of *pfmdr1-86* Gene

Briefly, the primers N1FP-*pfmdr86* (5′-AGGTTGAAAAAGAGTTGAAC-3’) and N1RP-*pfmdr86* (5′-ATGACACCACAAACATAAAT-3') were used in the first round of the nested PCR for the gene *pfmdr1-86*. 0.625 µl of each 0.25 M forward and reverse primer, 5 µl of 1X polymerase buffer, 2 µl of 25 mM MgCl2, 0.25 µl of 0.25 Mm dNTPs, 0.2 µl of 1 u/ µl Taq Polymerase, and 3.0 µl of DNA template were utilized in the main PCR reaction mixture of 25.0 l. In a thermal cycler (VWR) (Schmidt, Germany), PCR was carried out. Pre-denaturation was carried out at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 sec, 56 °C for 45 sec, and 72 °C for 1 min, and finally final elongation was carried out at 72 °C for 10 min. Nested PCR was then performed after the initial PCR. For the second round of the nested PCR, the forward N2FP-Pfmdr86 (5′ACAAAAAGAGTACCGCTGAAT3') and reverse primers N2RP-Pfmdr86 (5′AAACGCAAGTAATACATAAAGTC-3’) were used in turn. 0.625 µl of each 0.25µM forward and reverse primer, 5µl of 1X polymerase buffer, 2µl of 25mM MgCl2, 0.25µl of 0.25Mm dNTPs, 0.2 µl of 1u/µl Taq Polymerase, and 2.0 µl of primer PCR product were used in the amplification reaction volume of 25µl. The nested PCR was carried out using the following protocol: pre-denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 30 sec, 48 °C for 45 sec, and 72 °C for 1 min, and finally elongation at 72 °C for 10 min.

3.8.5. *pfcrt K76T* and *pfmdr1 N86Y* Codons Genotyping by RFLP

3.8.5.1. *pfcrt K76T* Codon Genotyping by RFLP

Site-specific restriction enzymes (New England Biolabs) were used to subject the PCR amplicon to restriction fragment length polymorphism. By allowing the restriction enzyme Apo I to sit in the amplicon at 50°C for 5 hours, the *pfcrt76* alleles were examined. As this would mimic typical circumstances in malaria settings, nested PCR products were employed without purification processes (Djimde *et al*., 2001; Veiga *et al*., 2006). The digestion restriction was carried out in accordance with the manufacturer's instructions. The digesting conditions will be as stated previously, and restriction enzymes were utilized (UMSM, 2012). In 20.0 µl of reaction mixture, including 12.8 µl of nuclease-free water, 0.2 l of 1u/l ApoI restriction enzyme, 2.0 l of 1X NEBufer r3.1, and 5 l of nested PCR products, restriction

enzyme digestion was carried out. In the processes, known positive controls (Pf3D7, wild-type and Dd2, mutant type) and nuclease-free water (negative control) were used in place of the nested PCR product. The *pfcrt 76T* (145 bp) allele is not cleaved by Apo I restriction enzyme, whereas the *Pfcrt 76K* allele is cleaved into two fragments (99 & 46 bp) determined by 100 bp ladder. After being electrophoresed on a 2.0% agarose gel, the cleaved PCR products (bands) of the *pfcrt K76T* codon were then visible with a UV trans-illuminator. Restriction enzyme digestion was done in 20.0 µl of reaction mixture containing 12.8 µl nuclease-free water, 0.2 µl of 1u/µl ApoI restriction enzyme, 2.0 µl of 1X NEBufer r3.1, and 5 µl nested PCR products. Restriction enzyme was used, and the digestion conditions were as previously described (UMSM, 2012). Known positive controls (Pf3D7, wild-type, and Dd2, mutant type) and nuclease-free water (negative control) instead of the nested PCR product were included in the reactions. The enzyme cuts *pfcrt 76K* into two fragments (99 bp and 46 bp) but not the *pfcrt 76T* (145 bp) allele detrmined by 100 bp. Cleaved PCR products (bands) of the *pfcrt K76T* codon were then visualized with a UV trans-illuminator after electrophoresis using a 2.0% agarose gel.

3.8.5.2. *pfmdr1-N86Y* Codon Genotyping by RFLP

Site-specific restriction enzymes (New England Biolabs) were used to subject the PCR amplicon to restriction fragment length polymorphism. The restriction enzymes Apo I and Afl III were incubated with the amplicon at 50 ^{o}C for 5 hours and at 37 ^{o}C overnight to investigate the *pfmdr1N86Y* allele respectively. As this would represent typical circumstances in malaria settings, nested PCR results were employed without purification processes (Djimde *et al.*, 2001; Veiga *et al.*, 2006). The digestion restriction was carried out in accordance with the manufacturer's instructions. Restrictions enzymes were used, and the digestion conditions were as previously described (UMSM, 2012). It took 20.0 µl of reaction mixture, 12.8 µl of nuclease-free water, 0.2 µl of each ApoI and Afl III restriction enzyme individually, 2.0 µl of 1X NEBufer r3.1, and 5 µl of nested PCR products to complete the restriction enzyme digestion. Instead of the nested PCR product, the procedures also contained known positive controls (Pf3D7, wild-type, and Dd2, mutant type) and nuclease-free water (negative control). The enzyme Apo I cuts the *pfmdr1-N86* allele (wild type) into three fragments (250 bp + 203 bp + 73 bp), while the *pfmdr1-86Y* allele (mutant type) is cut

into two fragments (454 bp + 73 bp). The enzyme Afl III cuts *pfmdr1-86Y* into two fragments (353 bp and 181 bp) but not the *pfmdr1-N86* allele (wild type; 534 bp). Following 2.0% agarose gel electrophoresis, cleaved PCR products (bands) of the pfmdr1-N86Y codon were seen using a UV trans-illuminator. The combined band patterns of the digested and undigested products seen in each DNA sample were interpreted as representing mixed haplotypes, which contain both wild and mutant types.

3.8.6. Agarose Gel Electrophoresis

The results of amplification were seen on a 2.0% agarose gel that had been stained with ethidium bromide. A 2.0% agarose gel was ready by dissolving 2.0 grams of multipurpose agarose in 100 ml of 0.5 x TBE buffer and boiling until the agarose melted and became uniform. Then, After carefully mixing 3µl of ethidium bromide into the melted agarose gel, the mixture was cast onto the gel and allowed to solidify. Once the gel had solidified, the comb was removed and the electrophoresis tray was inserted into the electrophoresis device, and until the gel was coated, 0.5 x TBE buffer was filled into the chamber. Then, 8µl of each nested PCR product was loaded into each sample well of the gel. Since the buffer was colored, there was no need for loading dye. A 100-bp DNA ladder was also included as a reference. Then, the gel was run at V = 120V, mA = 400, and time = 60 min for both *pfcrt76* and *pfmdr1-86*. Ultimately, the gel was taken out of the electrophoresis device and exposed to a trans-illuminator UV light source. The image was then recorded on a computer, labeled with the gel number, gene name, samples, and date. The remaining 17µl of nested PCR product was stored at -20oC for future use. Outer and nested PCR were repeated for negative samples after troubleshooting. The digested products were also subjected to electrophoresis, as were the nested PCR products.

3.9. Data Analysis

Data were initially input into Microsoft Office Excel® 2010 (Microsoft Corporation). After the data had been validated for completeness and consistency, Statistical Package for Social Science (SPSS) version 25 was used to analyze the cleaned data. To compare the prevalence of parasite densities between research locations, The Pearson's Chi-square test was performed using the Statistical Package for Social Science (SPSS) version 25. To examine the

prevalence differences across research locations, Fisher's exact test was used. To determine the distribution of the wild and mutant alleles between the study sites, pairwise comparison with the Bonferroni correction was utilized. Statistical significance will be determined by a P-value of 0.05.

3.10. Data Quality Control

In order for the survey's results to be reliable and valid, skilled lab professionals examined the blood sample following standard laboratory procedures at the health center following established protocol (WHO, 2010). Step-by-step data quality control was taken by the experimenter, and for each participant, a unique study code was used for a sample. In REDCAP, data from both the field and the lab were double-entered.

3.11. Ethical Consideration

The protocol for the study was reviewed and approved by the National Research Ethical committee (MoSHE/04/246/830/21) (Annex 1.8) and the Armauer Hansen Research Institute 's institutional ethics review committees (PO/46/20) (Annex 1.9). Initially, ethical clearance was obtained from each participating institution. A support letter was obtained from the Oromia, Amhara, and Gambella regional health bureaus and presented to each sample collection site hierarchically. Final protocol approval was obtained from the National Research Ethics Review Committee at the Federal Ministry of Science and Higher Education. Before obtaining samples from patients, the benefits, risks, and nature of the study for patients who meet the basic recruitment criteria were explained. Patients were informed about the study and offered the choice to participate or not through an informed consent process to allow for their blood to be taken. Participants in the study were made aware of the confidentiality of the data they collected. An informed, voluntary, written, and signed consent was obtained from each participant, and assent was obtained from parents or guardians of children less than 18 years old after the aim and nature of the study were fully explained to them. Giving permission and their participation in this study were fully voluntary. The participants were free to choose whether or not to take part in this research. They were free to leave the study at any moment, even if they chose not to participate; this did not entitle them to any advantages to which they would have otherwise been entitled.

4. Result

4.1. Demographic Characteristics of the Study Participants.

Overall, 258 individuals with falciparum malaria were enlisted to take part in the study. 83 from Abobo, 85 from Dera, 45 from Metemma, and 45 from Fentale. Of the 258 patients involved in the study, 164/258 (63.6%) and 94/258 (36.4%) were males and females, respectively. Between the ages of 2 and 68 were the participants, and 17.30±11.467 years was the mean age. The mean ages of the patients were 17.1, 15.55, 20.02, and 18.0 for Abobo, Dera, Metemma, and Fentale, respectively. The interquartile range was 8–24 years old, while the median age was 15. Of the 258 cases, 52.4% were above the age of 14 (Table 4).

Table 4. Demographic Characteristics of the Participants Recruited in the Study from four Sites in Ethiopia from July 2021 to October 2021 (n=258).

Variables	Abobo (N=83)	Metemma (N=45)	Dera (N=85)	Fentale (N=45)	Total (N=258)
Sex, n (%)					
Female	36 (43.4)	12 (26.7)	33 (38.8)	13 (28.9)	94 (36.4)
Male	47 (56.6)	33 (73.3)	52 (61.2)	32 (71.1)	164 (63.6)
Sub Total	83 (32.17)	45 (17.44)	85 (32.95)	45 (17.44)	258 (100)
Age(year)					
Median (IQR)	15(10–24)	20(12–25)	12(7–20)	18 (10–25)	15(8–24)
Mean (SD)	17.1 ± 10.34	20.02 ± 10.36	15.55 ± 13.55	18.24 ± 9.81	17.30± 11.47
Range	2–58	4–45	2–68	2–42	2–68
Age Category					
<5, n (%)	6 (7.2)	1 (2.2)	15 (17.6)	2 (4.4)	25 (9.7)
5-14, n (%)	31 (37.3)	15 (33.3)	36 (42.4)	17 (37.8)	98 (38.0)
15-30, n (%)	39 (47.0)	22 (48.9)	23 (27.1)	22 (48.9)	107 (41.5)
>30, n (%)	7 (8.4)	7 (15.6)	11 (12.9)	4 (8.9)	28 (10.9)
Sub Total, n (%)	83 (32.17)	45 (17.44)	85 (32.95)	45 (17.44)	258 (100)

IQR: interquartile range

4.2. Genomic DNA extraction

The genomic DNA of all microscopically confirmed samples (n=258) was extracted successfully with Magmax DNA extraction kit protocol.

4.3. Plasmodium parasites confirmation and quantification using *real-time qPCR*

Two hundred and fifty-eight extracted genomic DNA samples were successfully confirmed and quantified using a multiplex qPCR assay in relation to serial diluted of the NF54 cultured laboratory strain ranged from 10^6 to 10^3 copies/µL for *P. falciparum* and 10^7 to 10^4 copies/µL for *P. vivax* 18S plasmids. The representative figure below illustrates one *P. vivax* mono-infection, 4 mixed infections, and 71 *P. falciparum* mono-infection (Fig. 6). Based on the exclusion criteria, both *P. vivax* mono-infection and mixed infection samples were implemented, with substitution by confirmed *P. falciparum* mono-infection samples.

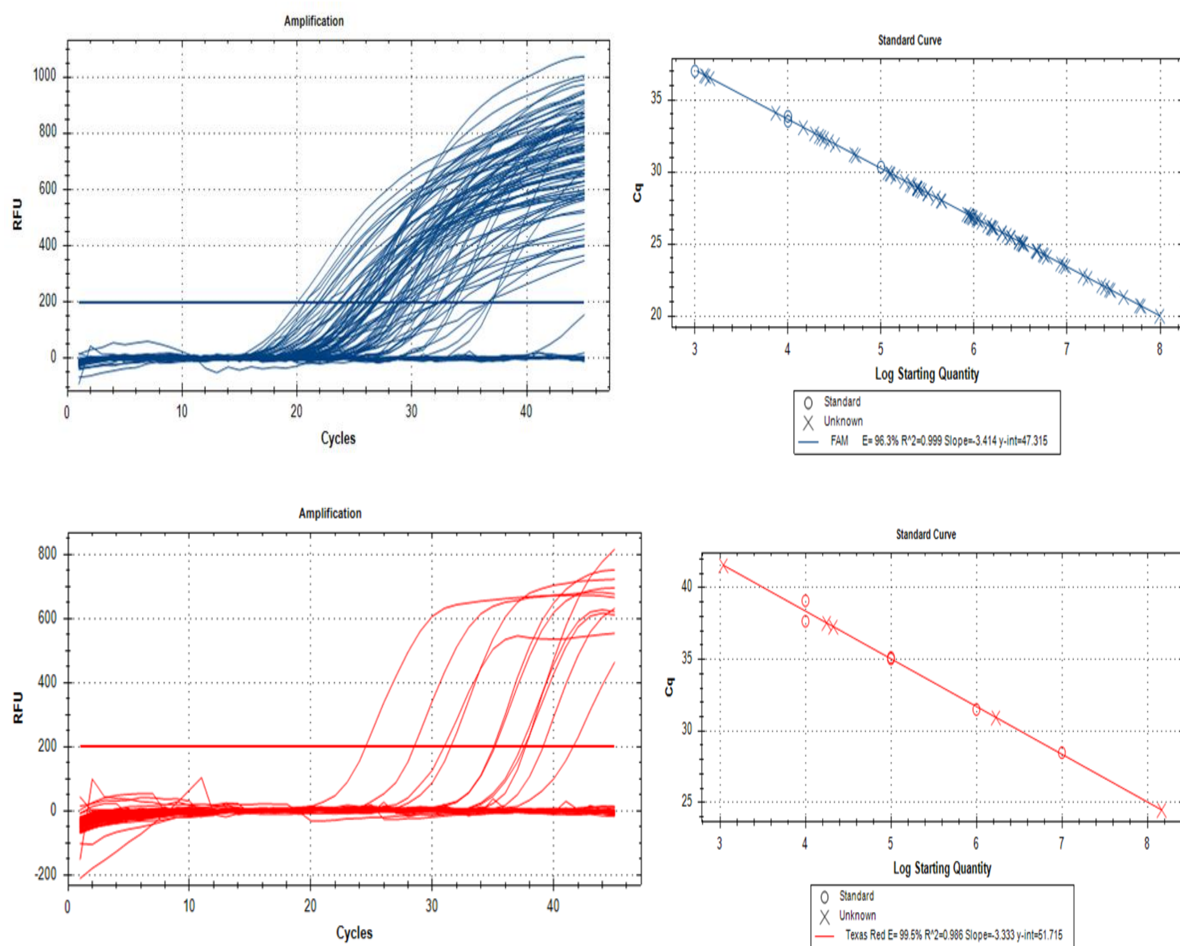


Figure 6 Amplification and quantification of *Plasmodium parasites* by multiplex qPCR Assay.

4.4. Parasitological Characteristics of the Study Participants

The parasite counts of the study participants ranged between 12–344,860 asexual stages/µl of blood, the geometric mean for parasitaemia being 2669.23. The median age and interquartile range were 2851.5 and 729–12,104 asexual parasites/µl, respectively (Table 5).

Table 5. Parasitological Characteristics of the Patients Recruited in the Study among from Four Sites in Ethiopia from July 2021 to October 2021 (n=258).

Variables	Abobo (N=83)	Metemma (N=45)	Dera (N=85)	Fentale (N=45)	Total (N=258)
Parasitaemia Level					
Range	12 – 344,860	207-49,869	114-51,081	33–112,248	12- 344,860
Median(IQR) parasites/µl	2,139(154–10,960)	2,699(921–7,136)	2,627(925.5–10,853)	10,256 (1,895 –24,120)	2851.5(729 - 12,104)
<1000,n (%)	36(43.4))	12(26.7)	21(24.7)	5 (11.1)	74(28.7)
1000 – 9999, n (%)	25(30.1)	24(53.3)	41(48.2)	17 (37.8)	107(41.5)
>10,000, n (%)	22(26.5)	9(20)	23(27.1)	23 (51.1)	77(29.8)

IQR: interquartile range

The geometric mean initial parasitaemia at the Abobo, Dera, Metemma, and Fentale sites were 1484.39 /µl, 3014.74 /µl, 2605.57 /µI, and 6412.85 /µl, respectively (Figure 7). Ultimately, there was a significant variation in the geometric mean parasitaemia.among the study sites ($\chi2$ = 26.25; P = 0.000).

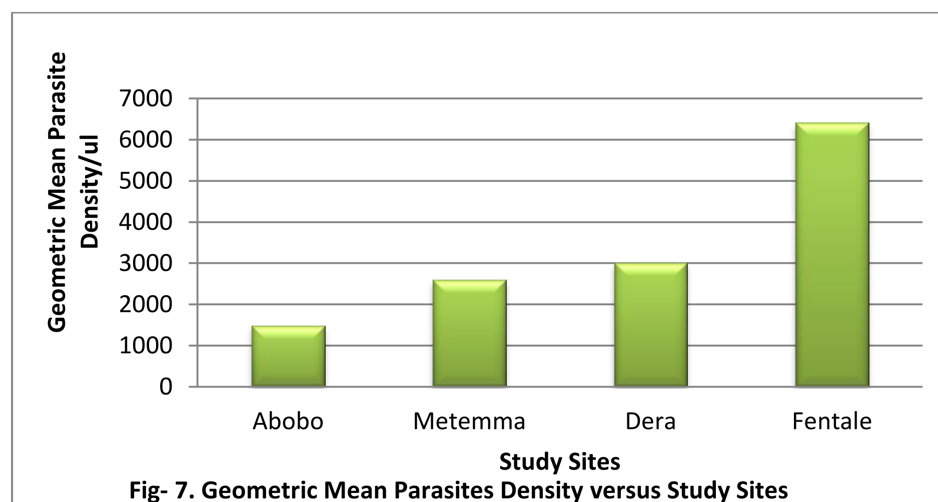


Fig- 7. Geometric Mean Parasites Density versus Study Sites

4.5. Prevalence of *pfcrt76* and *pfmdr1-86* Haplotypes

All 258 samples had effectively extracted DNA, as shown by the 18S real-time PCR analysis. In 250/258 and 257/258 samples, respectively, the *pfcrt76* and *pfmdr1-86* genes were effectively amplified, yielding genotyping success rates of 96.9% and 99.61%. Table 6 provides a summary of the examined SNPs' observed frequencies at the research sites' *pfcrt76* and *pfmdr1-86* loci.

In the districts of Dera (72/85, 84.71%) and Fentale (42/45, 93.33%), where *P. vivax* is extremely endemic (83.3% and 82.4% of cases, respectively), the mutant (pfcrt-76T) type was more prevalent (Awoke Minwuyelet et al., 2020; M Sleshi et al., 2012). However, Abobo (45/80 (56.25%) and Metemma (17/40 (42.5%) districts had greater incidence of the wild type (pfcrt-K76) codon (Table 6). In these conditions, the percentage of *P. vivax* infections is extremely low (less than 10%) (Getachew Ferede et al., 2013; Arega Tsegaye et al., 2014) (Table 6).

In 98.44% (253/257) of the examined samples, the *pfmdr1-86* gene containing the wild-type allele (N86) sensitive TGA nucleotide, coding for asparagine (Asn), was found. 1.56% (4/257) of the population had the mutant codon *pfmdr1-86Y*. About 31.2% (78/250) of the examined samples had the *pfcrt76* gene, which codes for the wild-type allele (K76) vulnerable AAA nucleotide and codes for lysine (Lys). In 68.8% of cases, the *pfcrt76* gene containing the mutant allele (76T) sensitive ACA nucleotide coding for threonine (Thr) was found. (172/250) (Table 6).

Of 78/250 (31.2%) *pfcrt-K76* of the analyzed samples, 56.25% (45/80), 42.5% (17/40), 15.29% (13/85) and 6.67% (3/45) were sampled from Abobo, Metemma, Dera, and Fentale, respectively. Of 68.8% (172/250) *pfcrt-76T*, 43.75% (35/80), 57.5% (23/40), 84.71% (72/85), and 93.33% (42/45) were sampled from Abobo, Metemma, Dera, and Fentale, respectively (Table 6). Furthermore, there were significant variations in the observed prevalence of the various haplotypes among the study's areas (P = 0.000) (Table 6). After Bonferroni correction, a significant difference in *pfcrt-K76* proportion was found between the study areas (P = 0.000).

In the districts of Dera, Metemma, and Fentale, the wild-type codon, *pfmdr1-N86*, was found to be fixed (100%) with comparable higher proportions in Abobo (95.18%; 79/83). 1.56% (4/257) of the population had the mutant codon *pfmdr1-86Y*. All the detected mutant codons were sampled from Abobo (4.81% (4/83)) (Table 6). There were significant variations in the observed prevalence of the various haplotypes throughout the study's locations (P = 0.025) (Table 6). Following the Bonferroni adjustment, there was not a significant difference in the percentage of *pfmdr1-N86* between the study's locations. (P = 0.255).

Table 6. Frequency Point Mutations of *pfcrt-76* and *pfmdr1- 86* Genes among Samples Recruited in the Study from Four Sites in Ethiopia July 2021 to October 2021 (n=258)..

Variables	Abobo (N=83)	Metemma (N=45)	Dera (N=85)	Fentale (N=45)	Total (N=258)	P-Value
Amplified *pfcrt-76*, n/N	80/83	40/45	85/85	45/45	250/258	
pfcrt-K76,% (n/N)	56.25 (45/80)	42.5(17/40)	15.29 (13/85)	6.67 (3/45)	31.2 (78/250)	
pfcrt-76T, % (n/N)	43.75 (35/80)	57.5(23/40)	84.71 (72/85)	93.33 (42/45)	68.8 (172/250)	0.000
Amplified *pfmdr1-86*,n/N	83/83	44/45	85/85	45/45	257/258	
pfmdr1-N86, % (n/N)	95.18(79/83)	100(44/44)	100(85/85)	100(45/45)	98.44(253/257)	
pfmdr1-86Y, % (n/N)	4.81 (4/83)	0.0 (0/44)	0.0 (0/85)	0.0(0/45)	1.56 (4/257)	0.025

n: the number of successfully amplified samples.

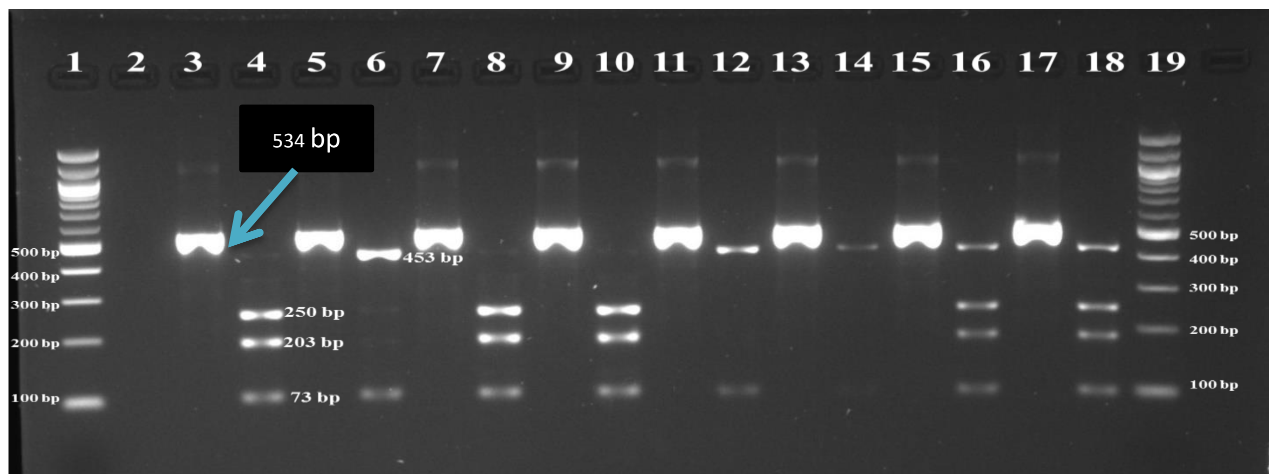


Figure 8 Representative samples photomicrograph of agarose gel showing the results of PCR- RFLP analysis of *pfmdr1-86* gene digestions with Apo I.
Lane 1 & 19- 100 bp Ladder, Lane 2- Negative Control, Lane 3- N2 Wild positive Control, Lane 4- Wild Positive Control Digested with Apo I, Lane 5- N2 Mutant Positive Control, Lane 6- Mutant Positive Control digested with Apo I, Lane 7 & 9- N2 Wild Samples, Lane 8 & 10- Wild samples Digested by Apo I, Lane 11 & 13- 2 Mutant Samples, Lane 12 &14- Mutant Samples Digested by Apo I, Lane 15 &17- N2 Mixed Samples, Lane 16 & 18- Mixed Samples Digested by Apo I.

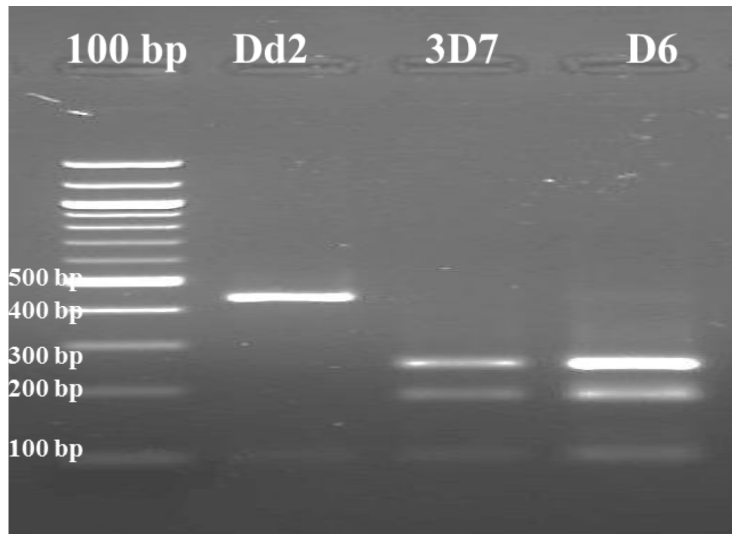


Figure 9 Positive controls *Plasmodium falciparum* laboratory strains (Dd2, 3D7 & D6) photomicrograph of agarose gel of PCR- RFLP analysis of *pfmdr1-86* gene digestions with Apo I.

Lane 1 - 100 bp Ladder, Lane 2- Mutant Positive Control (Dd2): Digested into Two Fragments (453 bp & 73 bp) with Apo I, Lane 3 & 4- Wild Positive Controls (3D7 & D6): Each Digested into Three Fragments (250 bp, 203 bp & 73bp) with Apo I.

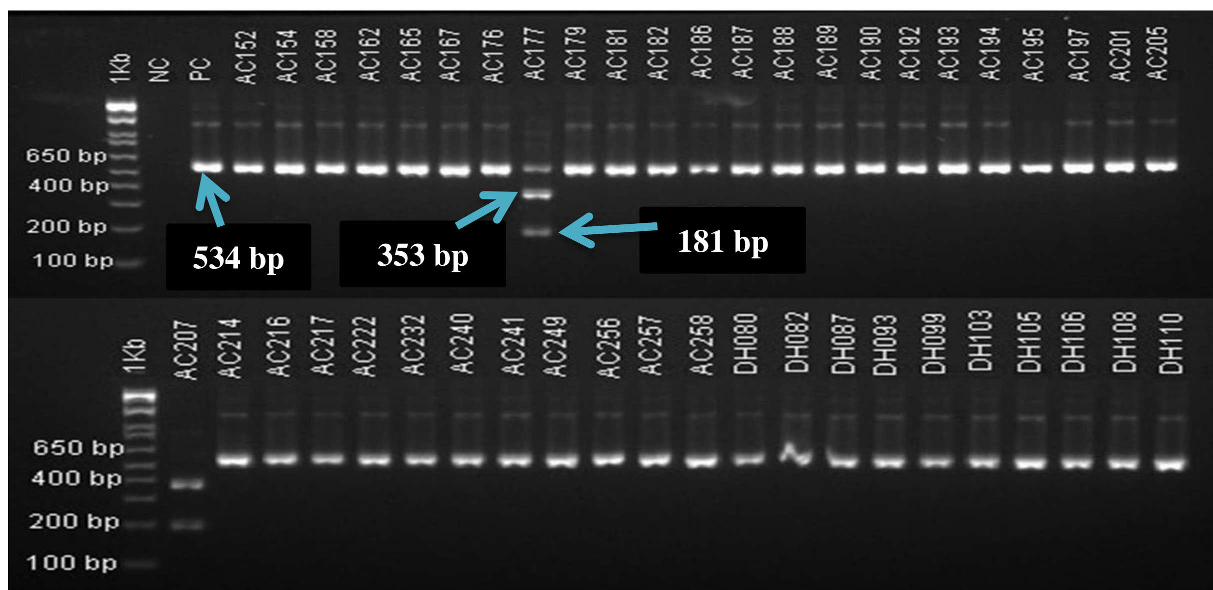


Figure 10 Photomicrograph of agarose gel showing the results of PCR- RFLP analysis of *pfmdr1-86* gene digestions with Afl III.

Lane 1 – 1kb bp Ladder, Lane 2- Negative Control, Lane 3- Wild Positive Control (undigested with Afl III), All samples except sample AC177 and AC207 were wild type (undigested). AC177- mixed sample digested by Afl III, AC207- mutant sample digested by Afl III.

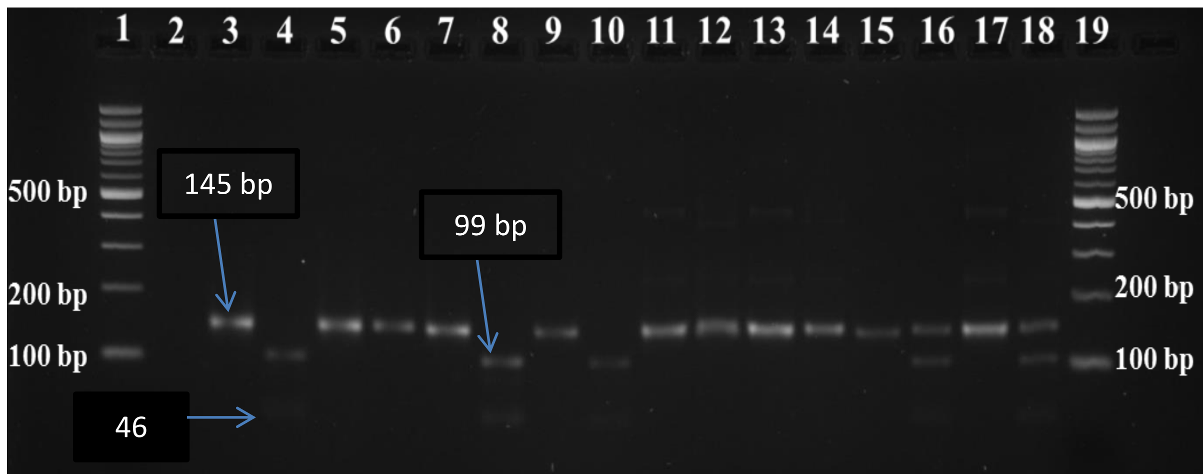


Figure 11 Representative samples photomicrograph of agarose gel showing the results of PCR- RFLP analysis of *pfcrt 76* gene digestions with Apo I.
Lane 1&19- 100 bp Ladder, Lane 2 - Negative Control, Lane 3 - N2 Wild Positive Control, Lane 4 - Wild Positive Control Digested with Apo-I, Lane 5 - N2 Mutant Positive Control, Lane 6 - Mutant Positive Control Digested with Apo-I, Lane 7 & 9 - N2 Wild Samples, Lane 8 & 10 - Wild Samples Digested with Apo-I, Lane 11 & 13 - N2 Mutant Samples, Lane 12 &14- Mutant Samples Digested with Apo-I, Lane 15 & 17- N2 Mixed Samples, Lane 16 & 18 – Mixed Samples Digested with Apo-I.

4.6. Prevalence of *Pfcrt 76T and pfmdr1-86Y* alleles by age groups per study site

Prevalence of *Pfcrt 76T* and *pfmdr1-86Y* alleles were assessed among age groups at Abobo, Metemma, Dera and Fentale sites. The *pfmdr1-86Y* alleles were detected only among 15–30 age groups with frequencies of 100 % (4/4). The highest and the lowest prevalence of *Pfcrt 76T* allele were detected among 5–14 age groups with frequence of 36.6% (63/172) and among 0-4 age groups with frequence of 12.8% (22/172) respectively (Fig. 12). However, no statistical signifcance difference of 76T and 86Y alleles were detected among age groups (Fisher's exact test, *P*=0.06) and (Fisher's exact test, *P*=0.186) for *pfcrt76* and *pfmdr1-86*.

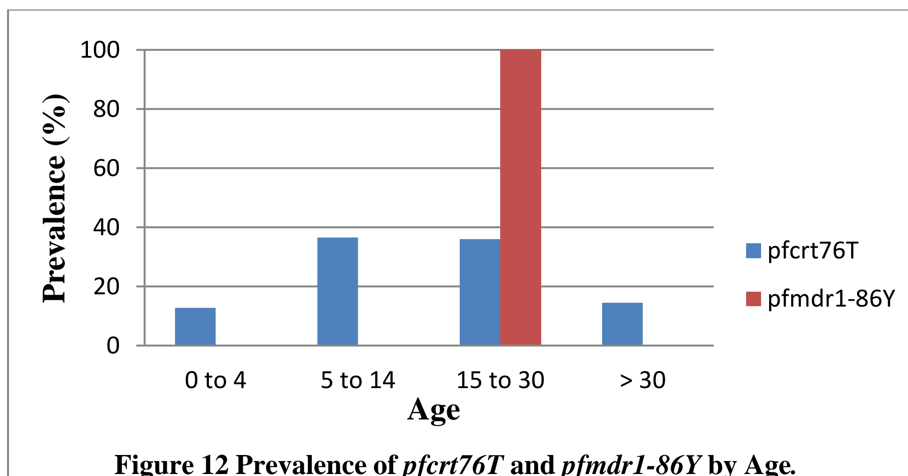


Figure 12 Prevalence of *pfcrt76T* and *pfmdr1-86Y* by Age.

The *pfmdr1-86Y* alleles were detected only among 15–30 age groups with frequencies of 100 % at Abobo. High prevalence of *Pfcrt 76T* alleles were detected among 15–30 age groups with frequencies of 40 % at Abobo, 47.8 % at Metemma and 47.6 % at Fentale but high prevalence of pfcrt76T allele at Dera was detected among 5-14 age groups with frequency of 38.9 % (Fig. 13).

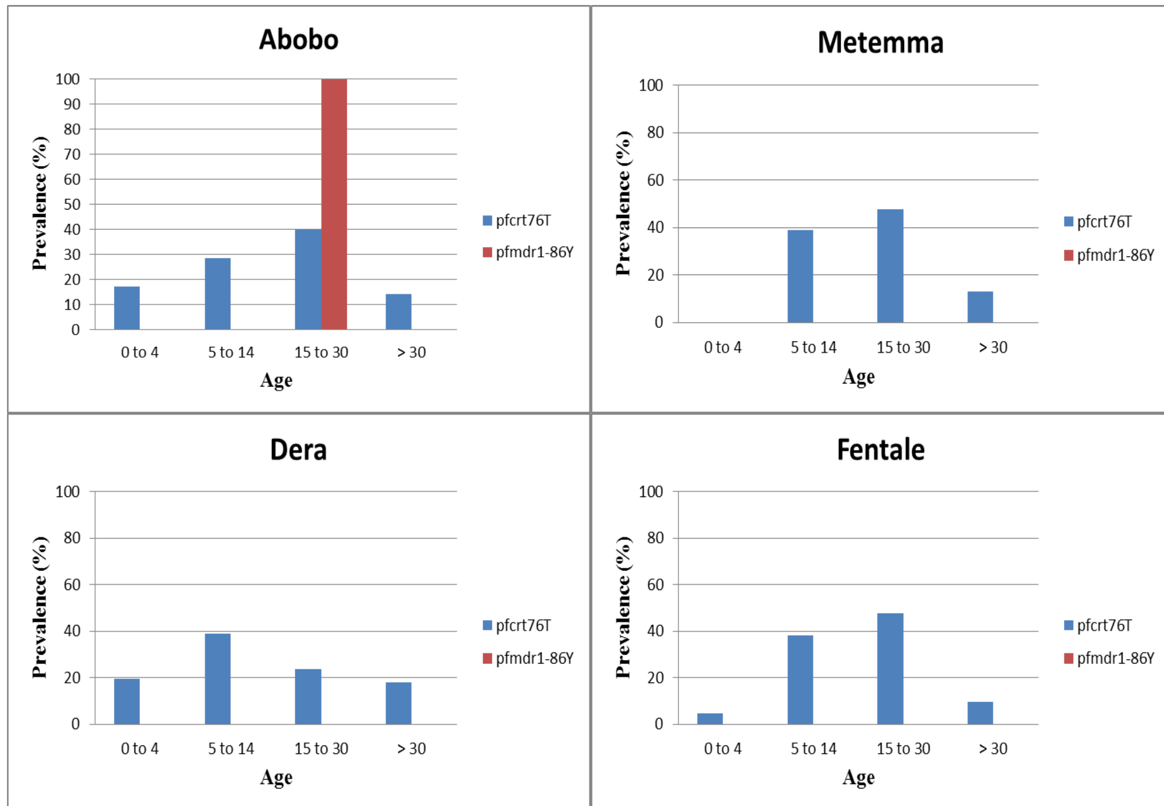


Figure 13 Prevalence of *pfcrt76T* and *pfmdr1-86Y* Alleles among Age Groups per Study Sites.

4.7. Prevalence of *pfmdr1-86Y* and *pfcrt 76T* allele by gender

Out of 257 and 250 isolates successfully genotyped for *Pfmdr1-N86Y* and *pfcrt K76T* codons, 63.42% (163/257) and 63.2% (158 /250) were males respectively. Females were more represented and contributed for higher prevalence 75% (3/4) of mutant *pfmdr1-86Y* allele (Fig. 14). However, the diference was not statistically signifcant (P= 0.198). While males were more represented and contributed for higher prevalence 64% (109/172) of mutant *pfcrt 76T* allele. However, the diference was not statistically signifcant (P=0.877).

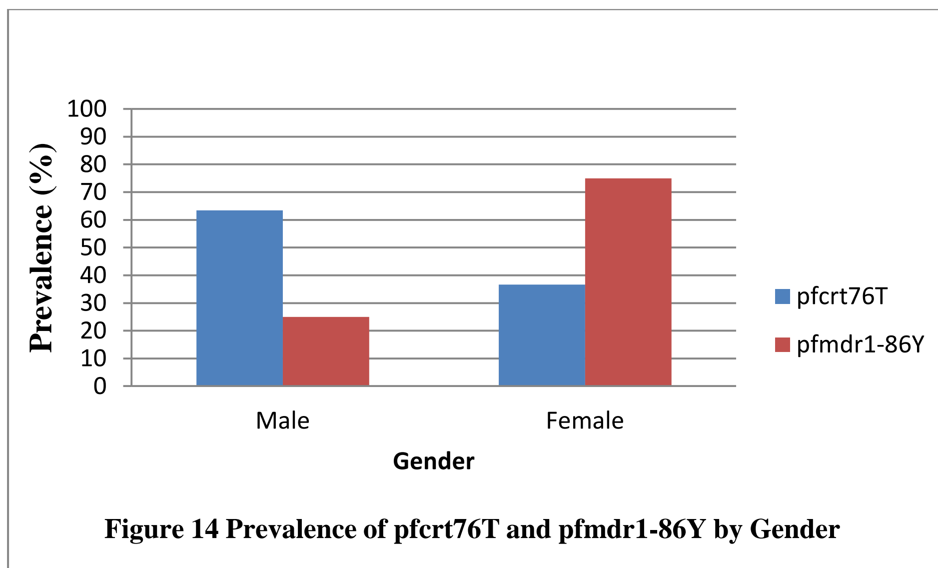


Figure 14 Prevalence of pfcrt76T and pfmdr1-86Y by Gender

5. Disscussion

Global public health is at risk from malaria, with a disproportionately higher burden of the disease in SSA (WHO, 2020). A significant global obstacle to the control of malaria is drug resistance (Koleala *et al*., 2015; Kumar *et al*., 2019). For some years, the largest obstacle in the fight against malaria has been P. falciparum's development of drug resistance to a number of anti-malarial drugs (Jifar Hassen et al ., 2022; Roux *et al*., 2021). A mutation in *pfcrt76T* is linked to chloroquine resistance, and a variant in *pfmdr1-86Y* may vary the degree of this resistance (Ngum *et al*., 2022). Due to the isolates' selective selection during the present regimen used to treat vivax and falciparum malaria in Ethiopia, CQ, and AL, respectively, there may be differences in the frequencies of mutant and wild type in the pfcrt 76 and pfmdr-1 86 codons (Lemu Golassa et al., 2015). Following the substitution of CQ by ACTs, it has been documented that CQ resistance genes' wild-type alleles have returned throughout Africa (Baraka *et al*., 2018; Elifaged Hailemeskel et al., 2019; Kavishe *et al*., 2014; Venkatesan *et al*., 2014). Due to the rapid transformation of *P. falciparum* from CQ-resistant to CQ-susceptible, it is possible that susceptible parasite populations will eventually replace CQ-resistance genes if CQ is stopped being used to treat P. falciparum or if pressure from AL, which has been used to treat falciparum malaria since 2004, is applied (Laufer *et al*., 2010; Mang'era *et al*., 2012; Seleshi Kebede et al., 2014). In order for malaria control efforts to be successful, ongoing surveillance is essential (Elifaged Hailemeskel et al., 2019). These indicators may be associated with ACT medication resistance. The purpose of the current study was to determine the prevalence of *pfmdr1-86* and *pfcrt 76* gene mutations in the *P. falciparum* population in Ethiopia under various levels of *P. vivax* endemicity.

Following the detection of a point mutation by PCR-RFLP in the *pfmdr1-N86Y* and *pfcrt-K76T* genes, CQ resistance (CQR) was monitored. Out of the 258 samples obtained at the four research sites, only 250 and 257 samples, respectively, amplified for the *pfcrt76* and *pfmdr1-86* genes in the PCR-RFLP analysis conducted for this study., Abobo, Metemma, Dera and Fentale. Some samples have failed to be amplified for *pfmdr1-86* and/or *pfcrt-76* as was the case for other studies (Adamu *et al*., 2020; Elifaged Hailemeskel et al., 2019; Jifar Hassen et al ., 2022). The amplification failure of some samples in this study might be

related to the strain nature or low parasitemia level as the optimized protocol well done for more than 90% of the samples.

P. vivax endemicity has been shown to be linked with a high frequency of the mutant pfcrt-76T allele (68.8% (172/250)), with rare examples of Dera and Fentale, as follows: 43.75% (35/80), 57.5% (23/40), 84.71% (72/85), and 93.33% (42/45) were sampled from Abobo, Metemma, Dera, and Fentale, respectively. Of the 250 *P. falciparum* isolates, only 31.2% (78/250) had the wild-type pfcrt K76 gene. Apart from a significant difference between the samples within the study areas (P = 0.000), differences were observed in the frequency of mutant and wild-type alleles at the pfcrt76 locus amongst samples within the study sites (P = 0.000). The proportion of *P. vivax* infections in the *P. falciparum* dominant area were 2.6% in Abobo (Tsegaye et al., 2014) and 9.0% in Metema (Getachew Ferede et al., 2013), while the proportion of *P. vivax* infections in areas where *P. falciparum* is co-endemic with high *P. vivax* were 83.3% in Dera (Awoke Minwuyelet et al., 2020) and 82.4% in Fentale (M Sleshi et al., 2012).

The observed variation in the reversal of mutations linked to CQ resistance may be explained by the difference in drug pressure in different parts of the country, as evidenced by the high levels of *P. vivax* co-endemicity in Dera and Fentale compared with dominance of *P. falciparum* infection in Abobo & Metema (Elifaged Hailemeskel et al., 2019; Geletta Tadele et al., 2023). Therefore, differences in pharmacological pressure and transmission intensities in each research location may account for the observed heterogeneity in the frequencies of mutant and wild-type variants for the *pfcrt76* loci in the study sites.

The higher prevalence of *pfcrt-76T* was observed in Fentale than in Dera, in contrast with the proportion of *P. vivax* infection; this might be due to a relative rise in *P. falciparum* prevalence, which is quickly taking over as the major species affecting the population in Dera, and a downward trend in *P. vivax* prevalence. There has been recent news of a change in the predominant malaria parasite species in the research area by Andargachew Almaw *et al.* (2022), where a prevalence of 23.08% for *P. vivax*, 58.65% for *P. falciparum*, and 18.27% for mixed were shown. This indicates that the dominant malaria parasite species in Dera has changed from the previous prevalence report, which served as the basis for the current study's

selection of the study site in 2021. Previously, *P. vivax* dominated the region (Tsegaye *et al.*, 2014), with a prevalence of 83.3% for *P. vivax*.

The high proportions of mutant *pfcrt 76T* alleles in Dera (84.71%) and Fentale (93.33%) are comparable with the study conducted in high *P. vivax* co-endemic areas in Ethiopia, such as Olenchiti (84.2%) and Fentale (92.7%) (Jifar Hassen et al ., 2022), respectively. On the other hand, the present prevalence of mutant *pfcrt 76T* alleles in Dera (84.71%) and Fentale (93.33%) is higher than other studies conducted in various parts of Ethiopia, such as Shewa Robit and Metehara (62.5%), Jimma and Halaba (54.8%), and Mankush and Bure (57%) (Lo *et al.*, 2017). On the other hand, the present prevalence of the mutant *pfcrt 76T* allele in Dera (84.71%) and Fentale (93.33%) is lower than other studies conducted in various parts of Ethiopia, such as Adama (100%) and West Arsi (100%) (Lemu Golassa et al., 2015), Babile (100%) and Adama (98.4%) (Elifaged Hailemeskel et al., 2019), Adama (95.7%) (Jifar Hassen et al ., 2022), West Arsi (100%) (Mula *et al.*, 2011), and Dilla (100%) (Schunk *et al.*, 2006). A comparable persisting high level of mutant *pfcrt 76T* allele has been reported from out of Ethiopia, such as Yemen (81.5%) (Al-Mekhaf *et al.*, 2011), Nigeria (74.3%) (Agomo *et al.,* 2021), Thailand (100%), Cambodia (100%), Laos (87%) (Iwagami *et al.*, 2018), and Gambia (76% in 2000) (Nwakanma *et al.*, 2014) and Thailand (99.1%) (Rungshirunrat *et al.*, 2009).

Although AL is the first line of therapy for *P. falciparum* in Dera and Fentale, there was a minimal recovery of parasites harboring wild-type K76. Since *P. vivax*, which causes approximately fourty percent of malaria cases in Ethiopia, is treated with CQ, the persistence of the high frequency of the 76T allele in Dera and Fentale may be linked to the drug's partial removal. It has already been shown that *P. vivax* dominates *P. falciparum* in Dera and Fentale, which may be a factor in the high frequency of the 76T allele (Lemu Golassa and Michael T, 2017; Elifaged Hailemeskel et al., 2019; Jifar Hassen et al ., 2022). Since research has indicated that the propagation of resistance has reduced in locations where drug consumption has declined, the survival of the mutant genotype, pfcrt 76T, in Ethiopia may be explained by the lack of total CQ withdrawal (Lemu Golassa and Michael T, 2017). CQ was largely removed from use in Ethiopia but is remains the recommended drug for treating uncomplicated *P. vivax* cases. Complete drug withdrawal is necessary in order to substitute

the less-fit CQ resistant genotype (Ochola *et al*., 2010). Because *P. vivax* treatment in the country is based on CQ, the at-risk population in high *P. vivax* co-endemic areas continues to unintentionally expose *P. falciparum* to CQ pressure.

The gradual reemergence of parasites with *pfcrt 76K* gene that are susceptible to CQ in Abobo (56.25%) and Metemma (42.5%) is higher than in Dera (15.29%) and Fentale (6.67%). The *pfcrt 76K* prevalence in Abobo and Metemma is comparable with the studies conducted in *P. falciparum*-dominant areas in Ethiopia, such as Gambella (58.5%) (Elifaged Hailemeskel et al., 2019) and Dembia and Gendawuha (45.11%) (Addimas Tajebe *et al*., 2015), respectively. On the other hand, the present prevalence of the wild *pfcrt 76K* allele in Abobo (56.25%) and Metemma (42.5%) is higher than other studies conducted in different parts of Ethiopia, such as Benshangul (34.8%) (Elifaged Hailemeskel et al., 2019).

On the other hand, the present prevalence of the wild *pfcrt 76K* allele in Abobo (56.25%) and Metemma (42.5%) is lower than other studies conducted in various parts of Ethiopia, such as Benshangul and Assosa (64.52%) (Geletta Tadele et al., 2023). The idea that the elimination of pharmacological selection pressure from chloroquine may encourage the resurgence of chloroquine-sensitive wild-type parasites is supported by the persistence of the wild-type genotype, *pfcrt 76K*, in the high *P. vivax* prevalence region of Ethiopia (Frosch *et al*., 2014). Treatment with artemether-lumefantrine, which is widely used in East Africa, has been shown to select for the pfcrt 76K variant that is sensitive to chloroquine. This suggests that the K76T mutation may be a drug-specific factor that contributes to increased sensitivity of P. falciparum to lumefantrine (Maiga *et al*., 2021; Sisowath *et al*., 2009).

In samples from *P. falciparum*-dominating areas, Gambella, and Metemma, there were fewer parasites with the mutant allele (*pfcrt 76T*). In areas where malaria transmission is prevalent, drug-sensitive and drug-resistant parasites compete, leading to a reduction in the proportion of *pfcrt 76T* in areas where P. falciparum is predominant (Elifaged Hailemeskel et al., 2019; Mulenga *et al*., 2021; Yobi *et al*., 2020). Jifar Hassen et al. (2022) report that SSA countries, including Malawi, Tanzania, Kenya, and Uganda, have shown a steady drop in the 76T allele and recovery of the 76K gene.

P. falciparum-dominated areas such as Abobo (56.25%) and Metemma (42.5%) were found to possess a significantly greater frequency of the pfcrt 76K allele in the wild type than high *P. vivax* co-endemic areas such as Dera (15.29%) and Fentale (6.67%). An encouraging step toward switching from the pricey AL to a secure and affordable form of CQ combined with additional fast-acting drugs is the re-emergence of chloroquine-susceptible alleles in an endemic malarial country such as Ethiopia. In nations where malaria is prevalent, even partial recoveries of chloroquine sensitivity are advantageous to public health (Laufer *et al*., 2010; Seleshi Kebede et al., 2014).

In the present finding a high frequency of the wild *pfmdr1 N86* allele (98.44% (253/257)) in the study areas was documented. *pfmdr1 N86* was found in higher proportion (95.18–100%) at all study areas; fixed in all except in Abobo (95.18%). After Bonferroni correction, No significant difference existed in the samples within the study's areas in the frequencies of wild-type and mutant alleles at the *pfmdr1-86* loci (P = 0.255). The frequencies of wild-type and mutant alleles at *pfmdr1 86* loci exhibited a significant difference among samples within the study areas (P = 0.025). This study's findings oppose earlier research from Ethiopia that found the *pfmdr1 Y86* mutation in *P. falciparum* to be present in 81% of cases (Schunk *et al*., 2006) and 84.5% of cases (Teferi Eshetu et al., 2010). This should be obvious given the significant gap in time between these two investigations and the current research, as well as the possibility that the easing of drug pressure on CQ over time may have helped to restore CQ susceptibility.

However, the high proportions of wild *pfmdr1 N86* allele (95.18–100%) in this study is comparable with the studies conducted in Ethiopia such as West Arsi (98%) (Lemu Golassa et al., 2015), Adama (100%), Olenchiti (100%) and Metehara (100%) Jifar Hassen et al ., 2022), Gambella (90.4%), Benishangul-Gumuz (77.3%), Babile (100%) and Adama (100%) (Elifaged Hailemeskel et al., 2019), north, east and south Ethiopia (above 80%) (Lo *et al*., 2017), Southern and Eastern Ethiopia (85.1%) (Seleshi Kebede et al., 2014), West Arsi (67.1%) (Mula et al., 2011) and Assosa (77.03%) and Gida Ayana (86.67%) (Geletta Tadele et al., 2023).

The high frequency of the *pfmdr1 N86* allele that is CQ-sensitive indicates that, in the AL presently used to treat falciparum malaria in Ethiopia, the wild type has a survival advantage over its corresponding mutant forms, as demonstrated by AL's choice of this allele elsewhere (Baliraine *et al*., 2011; Lemu Golassa et al., 2015). There is evidence linking this N86 allele to a decreased sensitivity to lumefantrine (Malmberg *et al*., 2013; Veiga *et al*., 2016), whereas prior research indicated chloroquine resistance in the 86Y gene. The *pfmdr1-N86 strain* selection, which lowers lumefantrine susceptibility of the parasite in study area, has been caused by the repeated use of AL treatment (Geletta Tadele et al., 2023), which might ultimately result in resistance (Otienoburu *et al*., 2016; Sondo *et al*., 2016).

Given that CQ is a chemical that is easy to manufacture, long-lasting, safe, and reasonably priced, after CQ resistance declines, the reemergence of P. falciparum that is susceptible to CQ will have consequences. The wild allele's *pfmdr1-N86* codon nearly complete reversion, confirms the hypothesis that ACT-based therapy may be to blame for this phenomena across all research locations (Seleshi Kebede et al., 2014).

The study's 15–30 and 5–14 age groups showed a high frequency of *pfmdr1–86Y* and *pfcrt76T* mutations, respectively. Nevertheless, there was no discernible relationship between the patients' ages and the K76T and 86Y mutation, which is in line with research conducted by Jifar Hassen et al. (2022), Atrosh et al. (2012), and Edogun et al. (2019). In line with Jifar Hassen et al.'s results, this study also found no significant difference between gender and the 76T and 86Y mutations (2022).

The small sample size for Metemma and Fentale examined due to logistical constraints is one of the study's shortcomings. We issued the mixed haplotypes, which were reported together with the mutant proportions. Because the analysis was based only on the PCR-RFLP method, it was impossible to determine if either of the 2 haplotypes originated from the same genome or from multi-clonal infections. *pfcrt 76* (18%, 45/250) and *pfmdr1 86* (1.17%, 3/257) mixed alelles were reported. Target gene copy number variations and infection clonality were not assessed.

6. Conclusion and Recommendation

6.1. Conclusion

In diverse settings of *P. vivax* co-endemicity areas in Ethiopia, this study displayed the percentages of *pfcrt K76T* and *pfmdr N86Y* molecular markers associated with resistance to CQ and AL antimalarial medications in individuals with symptoms of malaria. Even if it is no longer recommended for uncomplicated falciparum malaria in Ethiopia, CQ is still the cornerstone of treatment for vivax malaria. This might have had a role in the genotype's formation of CQ resistance in the Ethiopian study areas. After 20 years of using AL instead of CQ for the treatment of uncomplicated falciparum malaria in Ethiopia, the *pfcrt K76* allele is slowly but site-specifically reversing. The *pfmdr1 N86* wild type, on the other hand, remained essentially constant throughout the research locations. The resurgence of CQ-sensitive parasites is probably due to either the withdrawal of CQ from falciparum malaria treatment, the AL drug pressure since 2004, or both.

6.2. Recommendation

The researcher forwards the following recommendations based on the above findings:-

- To further recognize the CQ susceptibility recovery patterns and the efficacy of treating uncomplicated falciparum malaria with an AL-based treatment, it is recommended that further molecular epidemiological studies be conducted in different endemic locations with different histories of CQ exposure based on malaria parasites prevalence findings.
- Monitoring of molecular indicators of drug resistance in various settings of *P. vivax* endemicity throughout the country should continue to evaluate resistance dynamics and choose or adjust the most effective course of action.
- To make the more thorough investigation, infectious clonality, and copy number variation with PCR-RFLP assessment of antimalarial drug markers are recommended.
- Several locations with different histories of CQ exposure should be recruited for molecular surveillance investigations of malaria resistance to drugs under a single national initiative to provide timely data.

7. References

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Annexes

Annex 1.1: Sample size determination

The estimated sample size for current cross sectional study was calculated using double proportion formula with an assumption of 95% confidence level and 80% power. Thus, the total calculated number of participants was as follow:

Where;

- = Type I error (level of significance)
- = type II error (= (power of the study)
  - power = the probability of getting a significant result

Note that: =**7.85**, when the power = **80%** and = **5%**.

That is, =0. 05 (two sided) ⇒ = 1.96 *(from Z-table)*

= (1- 0.8) = 0.2 (one sided) ⇒ = 0.842 *(from Z-table)*

Thus,

***Pfmdr1* distribution in the study sites.**

- ✓ Fentale District= 28.0% (7/25)(Seleshi Kebede et al.,2014)
- ✓ Abobo District=9.6%(5/52)(Elifaged Hailemeskel et al., 2019)
- ✓ Dera District=22.7%(5/22)(Elifaged Hailemeskel et al., 2019)
- ✓ Metemma District=22.7%(5/22)(Elifaged Hailemeskel et al., 2019)

The P value for pure pf endemic area (Abobo + Metemma=P_1) and the pv co-endemic area(Fentale + Dera=P_2) was calculated as follow:

- ✓ P1=(X1 + X2)/n1+n2=(5 + 5)/52+22=**0.135**
- ✓ P2=(X3 + X4)/n3+n4=(5 + 7)/22+25=**0.255**

Therefor

$$n = \frac{(1.96+0.842)^2\ (0.135(1-0.135)+0.255(1-0.255))}{(0.135-0.255)^2} = 167.25 \approx 168$$

So the total sample size was **168**. The samples (n=**168**) was allocated based on the *pfmdr1* gene CNV distribution of the *P. falciparum* dominant areas and high *P. vivax* co-endemic study sites as follow:

To allocate the sample for each study area

Where
P_i : total population in study area 1
P_T : total number of population in all study areas (4 study area)
n : total number of sample

CNV of *pfmdr1* gene distribution

- ✓ Dera= 42/65 = **64.6%**(Lo et al., 2017)
- ✓ Methara=46/72 = **63.9**%(Lo et al., 2017)
- ✓ Metemma=42/65 = **64.6**%(Lo et al., 2017)
- ✓ Abobo=38/62 = **61.3**%(Lo et al., 2017)

$P_{pure\ pf\ endemic\ area}= P_{Metemma} + P_{Abobo}$ =42+38/65+62=<u>0.63</u>

$P_{Co\text{-}endemic\ area}= P_{Dera} + P_{Methara}$ =46+42/65+72=**<u>0.64</u>**

$P_{total}= P_{pf\ dominant\ endemic\ area} + P_{Co\text{-}endemic\ area}$=0.63 +0.64=**<u>1.27</u>**

$N_{pf\ dominant\ endemic\ araea}$=ntotal x $P_{pf\ dominant\ endemic\ area}/ P_{total}$=168 x 0.63/1.27=83.328~**<u>83</u>**

- ✓ n Abobo = 83

$N_{co\text{-}\ endemic\ araea}=n_{total}$ x $P_{C0\text{-}endemic\ area}$/ Ptotal=168 x 0.64/1.27=84.67~**<u>85</u>**

- ✓ n Dera = 85

Annex 1.2: Questionnaire and Consent Form

Questionnaire: **Socio Demographic Information and Survey Case Report Form**

A. Participant Information

1. Patient ID: 2. Health Center: 3. Date of Visit:…/……/…..

4. Age (Years): 5. Sex:: ..Male ☐☐ Female 6. Kebele:…………….

B. Microscopy Examination

Field health facility

7. status: ☐Positive ☐ Negative ☐Not Done

8. Species ☐*P.falciparum*☐*P.vivax*☐Mixed Species

9. Parasite count (parasite per microliter)........... 10. Name of microscopist...............

Confirmation at HARI

11. status: ☐Positive ☐ Negative ☐Not Done

12. Species ☐*P.falciparum*☐*P.vivax*☐Mixed Species

13. Parasite count (parasite per microliter)........... 14. Name of microscopist...............

15. If positive to question 7 above, is treatment provided? ☐Yes ☐No

C. Sample Type Collected

16. Is EDTA sample collected? ☐Yes ☐No

17. Is DBS prepared? ☐Yes ☐No

18. Is consent for long term storage of blood samples taken? ☐Yes ☐No

E. Previous malaria infection and travel history

19. In the past 2 weeks, have you had a test for malaria? ☐Yes ☐No

20. If yes, what was the result if the test? ☐Positive ☐Negative ☐ Don't know

21. In the past 2 weeks, have you taken any medicine for malaria? ☐Yes ☐No

22. If yes, what was the anti-malarial medicine taken?

☐ACT ☐AL ☐Artesunate ☐Fansidar(SP)

☐Quinine ☐Panadol ☐Other ☐Unknown

23. Have you travel to another locality of the country in the past 30 days? ☐Yes ☐No

24. If yes, where did you travel to the past 30 days?

Country........... Region............. District...... City/Village....... .....Don't Remember

25. If yes, for how long you travelled in the past 30 days?

No of days……… from...../....../........ To...../...../......

Name and signature of data collector...

Consent Form

Certificate of consent for Adults (>18Years)

Survey ID:__

I have been invited to participate in a study that aims to better understand if the malaria parasite is changing over time in Ethiopia. I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this study.

Name of participant:_Signature:____Date:_(dd/mm/yyyy)

Long-term storage and future studies: I agree to allow the study team to store my blood sample for fu	If you agree, circle "YES," if you Do not agree, circle,, NO".	
	YES	NO
Name:	Signature	Date //

Witness' signature: *A witness' signature and the patient's thumbprint are required only if the patient is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.*

I have witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Thumb print of participant

Name of witness:____Signature:____Date:_(dd/mmm/yyyy)

Investigator's signature:

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Name of Investigator:_Signature:___Date:_(dd/mm/yyyy)

Certificate of consent from the child`s family/guardian (<12years)

Survey ID:___

I have been invited for my child`s participation in a study that aims to better understand if the malaria parasite is changing over time and affecting how well rapid diagnostic tests are working in Ethiopia. I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to my child to participate in this study.

Name of participant`s parent:_Signature:____Date:__(dd/mmm/yyyy)

Long-term storage and future studies: I agree to allow the study team to store my child' Name:	If you agree ,circle "YES, "if You do not agree, circle,, NO".	
	YES	NO
	Signature	Date//

Witness' signature: *A witness' signature and the participant's thumbprint are required only if the participant is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.*

I have witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Thumb print of participant

Name of witness_____Signature_____Date:__

Long-term storage and future studies: I have witnessed the accurate reading of the request f Participant agrees:	Ifyouagree,circle "YES," if you do not Agree , circle,, NO".	
	YES	

Investigator's signature:

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Name of Investbigator:______Signature:____Date:__(dd/mm/yyyy)

the child`s family/guardian (≥12<18years)

Survey ID:___

I have been invited for my child to participate in a study that aims to better understand if the malaria parasite is changing over time and affecting how well rapid diagnostic tests are working in Ethiopia. I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to my child to participate in this study.

Participant`s parent name:___Signature:____Date:__

Long-term storage and future studies: I agree to allow the study team to store my child's blood sample	If you agree, circle "YES" ,if you do not agree, circle„ NO".	
	YES	NO
Name:	Signature	Date //

Witness' signature: A witness" signature and the patient's thumbprint are required only if the patient is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.

I have witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Thumb print of participant

Witness name:Signature:____Date:__

Long-term storage and future studies: I have witnessed the accurate reading of the request for long term sto	If you agree, circle "YES, "if you do not agree, circle„ NO".	
	YES	NO

Investigator’s signature:

Ihaveaccuratelyreadorwitnessedtheaccuratereadingoftheconsentformtothepotentialparticipant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Name of Investigator:_Signature:____Date:__(dd/mm/yyyy)

Certificate of Assent from child (>12<18years)

I understand this research is to understand if the malaria parasite is changing over time and affecting the way malaria tests work. I understand that I will get a finger prick for two malaria tests today and a few drop son paper to use for the malaria research in the future.

I have read this information (or had the information read to me)I have had my questions answered and know that I can ask questions later if I have them. I agree to take partin the research.

Only if child assents:

Name of child_Signature of child:___Date:__

Long– term storage:

Long-term storage and future studies: I agree to allow the study team to store my blood sample for future stu	If you agree, circle “YES, ”if you do not agree, circle,, NO”.	
	YES	NO

Only if child assents:

Name of child_Signature of child:___Date:__

If illiterate:A literate witness must sign (if possible, this person should be selected by the participant, not be a parent, and should have no connection to the research team). Participants who are illiterate should include their thumb print as well.

Witness’ signature:I have witnessed the accurate reading of the assent form to the child, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Thumb print of participant

Witness name: Signature:____Date:__

Investigator's signature:

I have accurately read or witnessed the accurate reading of the assent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given assent freely.

Name of Investigator: Signature:____Date:__(dd/mm/yyyy)

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the child understands that the following will be done: (1). finger prick for malaria test and blood spotonfilterpaper(2).longtermstorageofthefilterpaperforfutureresearch

I confirm that the child was given an opportunity to ask questions about the study, and all the questions asked by him/her have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

Name of person taking the assent___Signature:____Date:__

Parent/Guardian has signed and informed consent__Yes___No____(Name of researcher/assistant

Annex 1.3: Amharic Version Questionnaire and Consent Form

I; የተሳታፊዎች መረጃ እና የስምምነት መግለጫ ቅጽ

I. የተሳታፊዎች መረጃ ቅፅ

እኔ **ታጁዲን አብዱራሀማን ሀምዛ** በአዲስ አበባ ዩኒቨርሲቲ በባዮቴክኖሎጂ ት/ት ክፍል የባዮቴክኖሎጂ የማስተርስ ተማሪ ስሆን የመመራቂያ ጽሁፌን በሰሜን ምህራብ እና በደቡብ ምህራብ ብሎም በመካከለኛው ኢትዮጵያ በሚገኙ በፒ.ፈልሲፋርምየወባ በሽታ አምጪ ተህዋሲየን በተጠቁ ህሙማን ላይ ምርምር ለማድረግ አቅጃለሁ። የዚህ ምርምር ውጤት የሚጠቅመው በመጀመሪያ ደረጃ የወባን በሽታ ቁጥጥርና የማስወገድ ፕሮግራም ላይ እንቅፋት የሚሆነውን ፀረ-ወባ መድሃኒት መቋቋም የጀመሩ የተዋህሲያኑን ስቴረን ስርጭታቸውን ለማወቅና ተገቢ የሆነ መፍተሄዎች እንዲወሰዱበት ነው። በመቀጠልም ተህዋሲያኑ ክሎሮኪን የተባለውን ፀረ-ወባ መድሃኒትን የመቋቋም ባህሪያቸው ከቀነሰ ኮአርትም የተባለውን ፀረ-ወባ መድሃኒት በአሁን ሰአት በተዋሲያኑ ለሚመጣ የወባ በሽታ እየተጠቀምነው ያለው ከክሎሮኪን ፀረ-ወባ መድሃኒት አንፃር በዋጋው ውድ በመሆኑ ክሎሮኪንን ከሌላ ፍቱንና ረከስ ካለ መድሃኒት ጋር በመቀላቀል እንደገና በጥቅም ላይ እንዲውል ለመጠቆም ነው።

አጠቃላይ መረጃ፡-

ወባ በፐላዝሞዲየም ዝርያ የሚመጣ ጥገኛ በሽታ ነው። የዓለም ጤና ድርጅት የአፍሪካ አህጉር ከ2000-2019 ዓ.ም ባለው ጊዜ ውስጥ 215 ሚሊዮን የሚገመቱ ጉዳዮች ሲኖሩ 94 በመቶውን ይይዛል። አምስት ጥ ገኛ ዝርያዎች በሰዎች ላይ የወባ በሽታ ያስከትላሉ፣ በኢትዮጵያ ከሚገኙ 109 ሚሊዮን ነዋሪዎች መካከል 2. 4 ሚሊዮን ግምት እና 962,000 የተረጋገጠ የወባ በሽታ በ2018 ተመዝግቧል። በሽታው የተያዙት አብዛኛዎ ቹ በፒ.ፈልሲፋርም የተከሰቱ ናቸው።

የጥናቱ ዋና አላማ፡-

የምርምሩም ዋና ዓላማ እንደ ሀገራችን ለፋልስፓረም ወባ በሽታ ህክምና ይውል የነበረው ክሎሮኪን የተባለው ፀረ-ወባ መድሐኒት ከተቋረጠ በኃላ ተህዋሲያኑ የሚያሳዩትን የመድሃኒት መቋቋም አቅም ስርጭቱ ምን ያክል እንደሆነ ለማወቅ ነው።

ጥናቱ ለተሳታፊዎች ያለው ጠቀሜታ፡

በጥናቱ ሚሳተፉ ፍቃደኛ ተሳታፊዎች ምንም አይነት የገንዘብ ክፍያ የለምነገር ግን በምርመራው ውጤት መሰረት የመታከም እድሉ ይኖርዎታል፡ በተጨማሪም የጥናቱ ውጤት በሰሜን ምህራብ እና በደቡብምህራብ ብሎም በመካከለኛው ኢትዮጵያ ያለውን የወባ ዘርያ ለመለየት እና በሽታ ውን ለመቆጣ ጠር ተጨማሪ ግብአት ይሆናል::በተዘዋዋሪ መንገድ ሌላ ህመምተኛ እንዲሁም ህብረተሰቡን የመጥቀ ም እድል ያስገኛል።

በጥናቱ ተሳታፊዎች ላይ ያለው ጉዳትና ተዛማጅ ችግር

በዚህ ጥናት በመሳተፍወ ሊደርስብዎ የሚችል አንድም ጉዳት አይኖርም፡ ለጥናቱ የደም ናሙና የሚያስፈል ግ ሲሆን ናሙና በሚሰጡበት ጊዜ ምንም አይነት ችግር አይገጥምዎትም፡፡

<u>የመረጃ ሚስጥራዊ አጠባበቅ</u>

ከእርስዎ ማህበራዊ መረጃ ጋር ተያይዞ የተገኘ እና ከእርስዎ ጋር ሊታወቅ የሚችል ማንኛውም መረጃ ሚስ ጥራዊ ሆኖ ይቆያል። በማንኛውም ጊዜ ጥናቱን የማቆም ወይም የመሰረዝ መብት አሎት። ስለእርስዎ የተሰ በሰበ መረጃ ቁጥሮችን በመጠቀም ኮድ ይደረጋል። የተሰበሰበ መረጃ ከጥናቱ ዋና ተመራማሪ ዉጪ ለሌሎ ች ሰዎች አይገለጽም ወይም በዚህ ጥናት ውስጥ በማንኛውም ዘገባ ላይ በቀጥታ አይታይም።

<u>ጥናቱን የሚያካሄደው ሰው ማረጋገጫ</u>

ለዚህ ጥናት ሃላፊነቱን ለመውሰድና ማናቸውንም ጥናቱ የሚመለከቱ ጉዳዮችን ክትትል ለማድረግና ለሚ መለከተው አካል መግለጫ ለመስጠት በፊርማዬ አረጋግጣለሁ፡፡

ስም፡ ታጁዲን አብዱራህማን

(አዲስ አበባ ዩኒቨርሲቲ፣ በባዮቴክኖሎጂ የት/ክፍል የሜዲካል ባዮቴክኖሎጂ ማስተርስ ተማሪ).

ፊርማ ------------------------------- ቀን ---------------------------

ማንኛውንም ጥያቄ መጠየቅ ለሚሹ የሚቀጥለውን አድራሻዬን መጠቀም ይችላሉ፡፡

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አማካሪዎች፡

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II የስምምነት መግለጫ ቅጽ

መለያ ቁጥር ________________

እኔ ከዚህ በታች ስሜ የተጠቀሰውና የፈረምኩት ግለሰብ በሰሜን ምዕራብ እና በደቡብ ምዕራብ ብሎም በመካከለኛው ኢትዮጵያ በሚገኙ ወባ በያዘአቸዉ ሰዎች ላይ እንደ ሀገራችን ለፋልስፓረም ወባ በሽታ ህክምና ይውል የነበረው ክሎሮኪን የተባለው ፀረ-ወባ መድሐኒት ከተቋረጠ በኃላ ተህዋሲያኑ የሚያሳዩትን የመድሃኒት መቋቋም አቅም ስርጭቱ ምን ያክል እንደሆነ መዳሰስ ስለማወቅ በሚደረገው ጥናት እና የጥናቱ አላማ የተነገረኝና ግልጽ የተደረገልኝ ሲሆን እንዲሁም ከኔ የተወሰዱት መረጃዎች በሙሉ በሚሰጥር የሚጠበቁ መሆናቸው ተነግሮኛል፡

፡ በተጨማሪም በማንኛውምሁኔታና ጊዜ ከጥናቱ ያለመሳትፍ መብት ተረጋግጦልኛል፡

፡ ስለዚህ በጥናቱ ለመሳተፍም ሆነ አስፈላጊውን ናሙናና መረጃ ለመስጠት ተስማምቻለሁ።

ስም____________________________________

ፊርማ___________________________________

ቀን____________________________________

III. የወላጆች ወይም የአሳዳጊዎች <u>የስምምነት መግለጫ ቅጽ</u>

መለያ ቁጥር __________

እኔ ከዚህ በታች ስሜ የተጠቀሰውና የፈረምኩት ግለሰብ በሰሜን ምዕራብ እና በደቡብ ምዕራብ ብሎም በመካከለኛው ኢትዮጵያ በሚገኙ ወባ በያዘአቸዉ ሰዎች ላይ እንደ ሀገራችን ለፋልስፓረም ወባ በሽታ ህክምና ይውል የነበረው ክሎሮኪን የተባለው ፀረ-ወባ መድሐኒት ከተቋረጠ በኃላ ተህዋሲያኑ የሚያሳዩትን የመድሃኒት መቋቋም አቅም ስርጭቱ ምን ያክል እንደሆነ ለመዳሰስ በሚደረገው ጥናት እና የጥናቱ አላማ የተነገረኝና ግልጽየተደረገልኝ ሲሆን እንዲሁም ከ ኔልጅ የተወሰዱት መረጃዎች በሙሉ በሚሰጥር የሚጠበቁ መሆናቸው ተነግሮኛል፡፡ በተጨማሪም በማንኛ ውም ሁኔታና ጊዜ ከጥናቱ ልጄን ያለመሳትፍ እና የማቋረጥ መብት እንዳለኝ ተረጋግጦኛል፡
፡ ስለዚህ በጥናቱ ልጀን ለመሳተፍም ሆነ አስፈላጊውን ናሙናና መረጃ ለመስጠት ተስማምቻለሁ፡፡

ስም_________________________________

ፊርማ________________________________

ቀን_________________________________

IV. አማርኛ ቃለ መጠይቅ

ሰላም

እኔ **ታጁዲን አብዱራህማን ሀምዛ** በአዲስ አበባ ዩኒቨርሲቲ በባዮቴክኖሎጂ ት/ት ክፍል የጤና ባዮቴክኖሎጂ የማስተርስ ተማሪ ስሆን የመመራቂያ ጽሁፌን በሰሜን ምህራብ እና በደቡብ ምህራብ ብሎም በመካከለኛው ኢትዮጵያ በሚገኙ በፒ.ፈልሲፋርምየወባ በሽታ አምጪ ተህዋሲየን በተጠቁ ህሙማን ላይ ምርምር ለማድረግ አቅጃለሁ። የዚህ ምርምር ውጤት የሚጠቅመው በመጀመሪያ ደረጃ የወባን በሽታ ቁጥጥርና የማስወገድ ፕሮግራም ላይ እንቅፋት የሚሆነውን ፀረ-ወባ መድሃኒት መቋቋም የጀመሩ የተዋህሲያኑን ስቴረን ስርጭታቸውን ለማወቅና ተገቢ የሆነ መፍተሄዎች እንዲወሰዱበት ነው። በመቀጠልም ተህዋሲያኑ ክሎሮኪን የተባለውን ፀረ-ወባ መድሃኒትን የመቋቋም ባህሪያቸው ከቀነሰ ኮአርትም የተባለውን ፀረ-ወባ መድሃኒት በአሁን ሰአት በተዋሲያኑ ለሚመጣ የወባ በሽታ እየተጠቀምነው ያለው ከክሎሮኪን ፀረ-ወባ መድሃኒት አንፃር በዋጋው ውድ በመሆኑ ክሎሮኪንን ከሌላ ፍቱንና ረከስ ካለ መድሃኒት ጋር በመቀላቀል እንደገና በጥቅም ላይ እንዲውል ለመጠቆምነው:: ስለሆነም እኔ እርስዎን ለጥናቱ ጠቃሚ መረጃዎችን እንደሚሰጡኝ በማመን ለዚህ ጥናት ተሳታፊ እንዲሆኑ መርጨዎታለሁ። ስለዚህ ፈቃደኛ ከሆኑ የተወሰኑ ጥያቄዎች ንእንዲመልሱልኝ ና አንድ የደምናሙ ና እንዲ ሰጡኝ እጠይቃለሁ እርስዎ የሚሰጡን ማንኛዉም መረጃ ሚስጥሩ የተጠበቀ ነው። የእርስዎ የደም ናሙና የላብራቶሪ ዉጤት ወባን የሚያመለክት ከሆነ አስፈላጊውን መዳሃኒት እነዲሰጥዎት እናደርጋለን።

	መለያ ኮድ-------------	ቀን…………………..
ተ.ቁ	የማህበራዊ እና በህሪነክ ጉዳዮች ቃለመ ጠይቅ	
1	ፆታ	**ሀ.** ወንድ ለ. ሴት
2	እድሜ	______________
3	የላብላቶሪ ውጤት	…………………………

የመረጃ ሰብሳቢው ስም ___________ ፊርማ __________________

የሱፐርቫይዘር ስም _____________ ፊርማ __________________

መረጃው የተሰበሰበበት ________ ቀን__________________

Annex-1.4: Standard Operating Procedure for gDNA Extraction from DBS Sample

This SOP focuses on how to extracts gDNA from DBS sample by using MagMAX™ DNA Multi-Sample Kit.

Standard Operating Procedure:	The MagMAX™ DNA Multi-Sample Kit to extract DNA from Dried Blood Spot (DBS) onKingfisher flex automated extractor
Developed by:	Part Number 4428202 Rev. B 08/2009

Reaction formats:

The KingFisher Flex automated extractor is a plate handling robot designed for automated, high-throughput processing of nucleic acid isolation chemistries. It uses an advanced technology in which magnetic rods move particles through the processing steps to provide efficient and reproducible purification of proteins, nucleic acids and cells from a variety of starting materials.

Time required

To obtain samples of purified genomic DNA Using the MagMAX™-96 DNA Multi-Sample Kit, it takes approximately 1 hour. But it doesn't include sample and reagent preparation times.

Material and Equipment Required

Table 1. MagMAX™ DNA Multi-Sample Kit (PN 4413022)

Component	5 × 96 (PN 4413022)	Storage condition
DNA Binding Beads, 10 mg/mL‡	1 × 8.5 mL	2 to 8 °C
DNA Elution Buffer 1	1 × 72 mL	Room temperature
DNA Elution Buffer 2	1 × 72 mL	
Elution Plate, 96-well	3	
Multi-Sample DNA Lysis Buffer	1 × 250 mL	
MicroAmp® Clear Adhesive Film	5 × 4 films	
PK Buffer	1 × 50 mL	
Processing Plate, 96-well, 1.2-mL	5	
Wash Solution 1 Concentrate§	1 × 75 mL	
Wash Solution 2 Concentrate#	1 × 250 mL	
Water, nuclease-free	2 × 50 mL	
Proteinase K, 100 mg/mL	1 × 4.3 mL	− 15 to − 25 °C
Ribonuclease A (RNase A), 1 mg/mL	1 × 2.7 mL	

Materials required but not supplied with the MagMax DNA Multi – Sample Kit.

Item	Location
Ethanol	Para lab
96-well (or thermo-mixer) if available; if not use Heat – block for Eppendorf 2 ml tubes	The heat block is available at Extraction room or at Molecular Lab.
Isopropanol (ACS reagent grade or equivalent)	Para lab
Lab equipment (such as pipettors, pipet tips, vortexer, microcentrifuge	Para lab and at Extraction room
Plate shaker	Para lab
KingFisher Flex 96 Deep Well Magnetic Particle Processor	Extraction room
KingFisher -96 Deep Well Tip Combs	Par lab
KingFisher -96 Deep Well Plates	Par lab
KingFisher -96 Standard Plates (Skirted PCR plate)	Par lab
Microcentrifuge tubes, 2-mL	Par lab

Sample preparation

Recommended inputs for compatible sample

Whatman® FTA® or SS 903 cards – 2 x 2 - mm punches

DNA yield

From two punches of the DBS, the extracted gDNA yield will be about 0.2 – 1 µg.

Procedure

Heated block was preheated to 60 to 65 °C.

Isopropanol was added to wash solution 1 concentrate.

Ethanol was added to wash solution 2 concentrate.

The solutions were stored at room temperature.

DNA binding bead mix was prepared for the sample extraction and storeed at room temperature.

Component	Volume (µL)	
	1 well	96 – well plat*
DNA Binding Beads (10mg/ml)	16	1616
Water, nuclease - free	4	404
Total (DNA binding bead mix)^	20	2020

The proteinase K digestion was performed:

PK buffer/enzyme mix was Prepared :

Component	Volume (µL)	
	1 well	96 – well plate*
Proteinase K Solution (100 mg/ml)	8	808
PK Digestion Buffer	42	4242
Total (PK buffer/enzyme Mix)	50	5050

b. 50 µL of PK buffer/enzyme mix was added to a well on a KingFishe-96 Deep Well Plate for each sample

The solution was mixed by pipetting up/down 5 to 7 times after each sample transfer.

c. The plate was sealed using a MicroAmp® Clear Adhesive Film,

Then the sealed plate was incubated for 20 minutes at 60 to 65 °C on a 96-well heated block.

d. The plate was removed from the heat source, and then carefully the cover was removed.

e The liquid was carefully transfered from each well of the plate to another well of the same plate

2. 100 µL of Multi-Sample DNA Lysis Buffer was added to each sample on the plate, sealed, and then shakeed at speed 8 for 3 minutes on a titer plate shaker.

3. The plate was removed from the shaker,

The cover was carefully removed, and then 20 µL of DNA binding bead mix was added to each sample on the plate. And then the plate was sealed, then shakeed at speed 7 for 3 minutes on the titer plate shaker.

4. The plate was removed from the shaker,

The cover was carefully removed, and then 120 µL of 100% isopropanol was added on the plate to each sample.

The plate was sealed, then shaked at speed 7 for 3 minutes on the titer plate shaker.

5. The plates were prepared for the KingFishe Flex magnetic particle Processor

Plate		Reagent	Volume per well (µL)	Plate type
ID	Position			
Binding	1	Lysate, isopropanol, and beads (from steps 1 to 4)	580	Deep well
Wash 1	2	Wash Buffer 1	150	
Wash 2	3	Wash Buffer 2	150	
Wash 3	4	Wash Buffer 2		
Elution	5	DNA Eltuion Buffer 1 for initial heated and elution DNA Eltuion Buffer 2 for equilibration Note: The instrument prompts you to add	75 75	Skirted PCR

STOPPING POINT. Use the purified samples immediately or store the elution plate for later use at 2 to 6 °C for up to 24 hours or at − 20 to − 80 °C for prolonged storage.

Annex-1.5. Procedure for Detection and Quantification of Malaria Parasites using Real-time qPCR

(Bio-Rad CFX96 Deep Well Touch Real-Time System)

Purpose: This procedure provides instructions for Bio-Rad CFX96 Deep Well Touch Real-Time System for malaria detection and quantification.

Scope: This procedure is applicable in AHRI parasitology laboratory to investigate presence and density of *Plasmodium* parasites i.e. *P. falciparum* and *P. vivax* in the human blood samples by real-time qPCR using 18S markers.

Background:

This document describes how to perform a qPCR method for identifying and quantifying *Plasmodia* spp. (*Pf* and *Pv*). qPCR (qRT-PCR) is a specialized technique that allows a PCR reaction to be visualized "in real time" as the reaction progresses. It allows quantification of minute amounts of DNA sequences in a sample. qPCR is a very sensitive method of measuring DNA with densities below the threshold of detection of conventional detection methods.

Responsibility and accountability:

It is the responsibility of the Laboratory unit coordinator to ensure the effective implementation and maintenance of this procedure.

Personnels assigned to perform this technique are responsible to understand and follow the procedure properly.

Principle:

Real-Time Polymerase Chain Reaction (qPCR) is a technique that monitors the progress of PCR reaction in real-time. It is based on the detection of the fluorescence produced by a reporter molecule. There are many different fluorescence markers (fluorophore-containing DNA probes) used in qPCR but one of the most common is TaqMan probe. It is a hydrolysis probe which bear a reporter dye, often fluorescein (FAM) at its 5' end and a quencher tetramethylrhodamine (TAMRA), attached to the 3' end of the oligonucleotide. As the taq-polymerase start to synthesize new DNA strand in the extension stage, it causes degradation of the probe by 5' end nuclease activity and the fluorescein is separated from the quencher as a result of which

fluorescence signal is generated. As this procedure continues, in each cycle the number of signal molecule increases, causing the increase in fluorescence which is positively related with the amplification of the target.

In general, Bio-Rad's CFX Dx real-time PCR amplification systems allow PCR quantification with standard curve, gene expression analysis, allelic discrimination, and end-point analysis.

Reagents and Supplies:

Reagents

70% alcohol, DNA away solution, PCR master mix reagents (TaqMan MM 2x buffer, 18S forward primers, 18S reverse primers and probes), PCR water, Pf18S and Pv18S serial dilutions (plasmids)

Supplies and materials

M-Tork, Fine tip markers, Gloves, Pipette tips (10µL, 20µL, 100µL, 200µL, 1000/1250µL), PCR plate,

PCR tubes, Plate sealer (Microseal® 'B' PCR Plate Sealing Film, Bio-Rad), 2 mL Eppendorf tubes

Equipment:

Rack for holding the Eppendorf and PCR tubes, Box (Bowel) to remove used tips, Dispenser

Pipettes (10µL, 20µL, 100µL, 200µL, 1000µL), Biosafety cabinet, PCR plate spinner, Tube spinner

Ice maker machine, CFX96 Touch™ Real-Time PCR Detection System

Environmental and safety controls:

All blood samples are potentially infectious. All the PCR master mixes should be prepared in sterile environment in the biosafety hoods. Please respect universal precautions and use standard safety precautions for handling and disposing of infectious materials. Wear disposable gloves

during the whole procedure.

Calibration: NA

Quality control:

To ensure the quality of this test result, quality control activities are involved including standardization of procedures. To check the performance of the test we use known negative (MQ) and positive (patient blood) and/or standards (plasmids). In addition, series of optimization experiments are done before starting the actual experiments.

NOTE: This assay is used to identify which samples should be included and/or excluded for downstream procedures. Any sample with a CT value greater than 34, should be excluded. It is recommend grouping all samples with a CT > 34 and performing separate amplicon PCRs and electrophoresis for these samples. If the electrophoresis yields positive results (e.g. positive and clear bands on gel) you may proceed with downstream procedures. *Samples with a CT > 34 have either very low and/or no parasite DNA.*

NOTE: *The negative controls must be negative (no Ct or above 40.0). The positive controls must be positive (designated by Ct value below 40.0). The test should be repeated if the NTC has a positive Ct value, or if the positive control yields no positive results.*

For more information, please see: Lucchi, N.W., et al., Molecular diagnosis of malaria by photo-induced electron transfer fluorogenic primers: PET-PCR. *PLoS One*, 2013. 8(2): p. e56677.

Procedure:

1. Take the master mix box to the master mix room.

2. Turn on the master mix safety hood and also put on the light.

3. Clean the master mix cabinet by using 70% ethanol followed by wiping away with M-tork.

Table1. Primers and probes used in Plasmodium genus and species detection and quantification

Target 18S rRNA of	Primer/ Sequence(5'-3')	Probe/ Sequence(5'-3')	References
P.falciparum	FAL-F/5'CTTTTGAGAGGTTTTGTTACTTTGAGTAA3' FAL-R/5'TATTCCATGCTGTAGT	FAM / 5'TGTTCATAACAGACGGGTAGTC	(Perandin et al
P. vivax	VIV-F/5'ACGCTTCTAGATTAATCCACATAACT3' VIV-R/5'ATTTACTCAAAGTAAC	HEX / 5'TTCGTATCGACTTTGTGCGCATT	(Perandin et al

4. Also clean all pipettes, tip boxes and rack by 70% ethanol. Use "DNA away" to clean pipettes in the MM room if we use them in other rooms.

5. Take out the master mix reagents from the fridge except the probes, then leave them and put on the rack to thaw for sometime.

Note: Withdraw the probes from the fridge after the other components are mixed.

6. **In between MM preparation activities, take out the extracted samples to be tested and serial dilutions/plasmids from -20oC and put them on the sample room to thaw.**
7. **Ready a 2 mL Eppendorf tube to mix all master mix components.**

8. **According to their order, add TaqMan MM 2x (buffer), species-specific Primer FW and Primer RV (P. falciparum/vivax FW and RV primers), nuclease free water (PCR-water, or MQ, or ddH2O) into the labelled tube.**

Note: While adding the components, mix them using the pipette by sucking up and down manner. Also, care has to be taken to not create bubbles. Switch off the light when we use the probe as it's light sensitive.

9. **Return all the primer and probe aliquots to the fridge.**
10. **Dispense the prepared MM (15µL in each well) into the PCR plate wells considering the duplicate plasmids, negative controls (MQ/NTCs) and number of target samples (unknown).**
11. **Switch off the light in the sample room as our mix contains probe that is light sensitive.**
12. **Dispense 5µL of the duplicate plasmids (standards), samples and MQs into the dispensed master-mix as of the plate layout.**
13. **After pipetting our PCR samples align the adhesive film (the Bio-Rad microseal® 'B' seals) to the plate so that all wells are covered tightly.**
14. **Spin the plate in the PCR spinner to remove all bubbles.**
15. **Turn on the master mix safety hood and also put on the light.**
16. **Clean the master mix cabinet by using 70% ethanol followed by wiping away with M-tork.**
17. **Also clean all pipettes, tip boxes and rack by 70% ethanol. Use "DNA away" to clean pipettes in the MM room if we use them in other rooms.**
18. **Take out the master mix reagents from the fridge except the probes, then leave them and put on the rack to thaw for sometime.**
    Note: Withdraw the probes from the fridge after the other components are mixed.
19. **In between MM preparation activities, take out the extracted samples to be tested and serial dilutions/plasmids from -20oC and put them on the sample room to thaw.**
20. **Ready a 2 mL Eppendorf tube to mix all master mix components.**
21. **According to their order, add TaqMan MM 2x (buffer), species-specific Primer FW and Primer RV (P. falciparum/vivax FW and RV primers), nuclease free water (PCR-water, or MQ, or ddH2O) into the labelled tube.**
    Note: While adding the components, mix them using the pipette by sucking up and down manner. Also, care has to be taken to not create bubbles. Switch off the light when we use the probe as it's light sensitive.
22. **Return all the primer and probe aliquots to the fridge.**
23. **Dispense the prepared MM (15µL in each well) into the PCR plate wells considering the duplicate plasmids, negative controls (MQ/NTCs) and number of target samples (unknown).**
24. **Switch off the light in the sample room as our mix contains probe that is light sensitive.**
25. **Dispense 5µL of the duplicate plasmids (standards), samples and MQs into the dispensed master-mix as of the plate layout.**
26. **After pipetting our PCR samples align the adhesive film (the Bio-Rad microseal® 'B' seals) to the plate so that all wells are covered tightly.**
27. **Spin the plate in the PCR spinner to remove all bubbles.**

28. Turn on the computer and then turn on the BIO-RAD by the switch at the back of the machine.
29. On the desktop of the computer, double click on the icon “Bio-Rad CFX Maestro” software.
30. Click on the “User-defined” run type button in the main software toolbar.
31. From the window that appears click on “Select Existing”, on Local Disc D then select “18S TaqMan”.

Check /adjust the real time PCR machine thermal cycler conditions on the Protocol window as follows:

Thermocycler conditions		
Temp	Time	Cycle
50 °C	2:00	1
95 °C	10:00	1
95 °C	0:15	45X
60 °C	1:00	

32. Click on “Next” and in the window that appears click on “Edit selected”. Insert all the information and indicate which wells are containing the standards, non-templates and unknown samples followed by selecting the fluorophores,
33. Click on the “Open lid” button located on the software’s “Start Run” tab to open the motorized lid.
34. Load samples in the “block” of the CFX96 Deep Well system, then click on “Close lid” to close the motorized lid.
35. Select our target gene as “18S”.
36. Start the reaction by clicking on “Start Run”, after filling all the required information for the reaction.
37. Once cycling is completed, check our results by adjusting the baseline thresholds for each species of Plasmodia.
38. Export the result to excel and save the excel workbook.
39. Remove the samples from the block and close the system.
40. Press the power switch on the back of the C1000 thermal cycler to power it down.

Calculation: Calculation required for master mix preparation (amount of each component calculated by the qPCR calculator –automatic) can be consulted from the Excel.

TaqMan Probe based MM calculator				
qPCR mix:	Conc (µM)	Final conc (nM)	1x	96

TaqMan MM 2x		2		1		x	10	1056.0
Primer FW	1		0	8	3	3	1.7	175.9
Primer RV	1		0	8	3	3	1.7	175.9
Probe	1		0	1	1	0	0.2	23.2
ddH2O	fill up to 10µL						1.4	152.9
Total MM (µL):							**15**	1584.0
Input (µL)							5	15.0
Final volume (µL)							**20**	
Quantity check for MM							15	

Result Reporting and Interpretation:

A successful real-time PCR experiment will have the following characteristics:

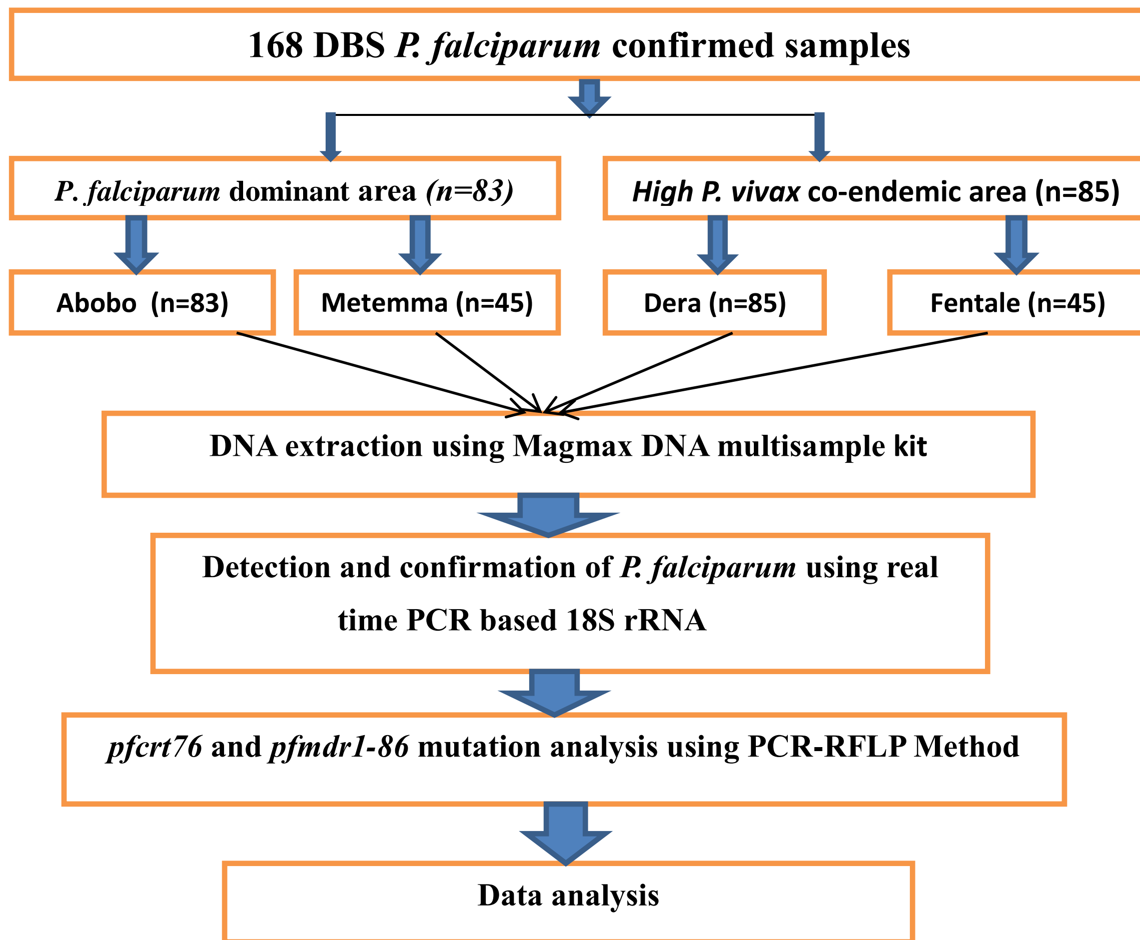
- Curves are all S-shaped
- Dilution series has expected spacing
- Replicates are tightly clustered
- Baselines are relatively flat
- Plateau height doesn't matter
- Curves are smooth
- Melt curve has one peak per product
- No signal for NTC (non-template control).

In general, slopes between -3.1 and -3.6 giving PCR efficiencies between 90 and 110% are typically acceptable.

References:

1. Nolan T, Huggett J, Sanchez E. Good practice guide for the application of quantitative PCR (qPCR), LGC. 2013.

Annex 1.6. Diagram Showing Work flow of the Study



Additionally **45** samples collected from Fentale representing the high *P.vivax* co-endemic areas and **45** samples collected from Metemma representing ***P. falciparum* dominant** endemic areas were used to strengthn the study.

Annex 1.7. National Research Ethical Committee Approval Letter

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Ministry of Science and Higher Education - Ethiopia

Ref.No. MoSHE/04/246/830/21
Date: 29 JUL 2021

Armuer Hansen Research Institute (AHRI)
Addis Ababa

Subject: *Letter of Approval*

The Ministry of Science and Higher Education (MoSHE) via its National Research Ethics Review Committee has reviewed"**African Centre for hrp2/3 Deletion Surveillance (ACHIDES) –Ethiopia''** Project protocol in an expedited manner. We are writing to advise you that MoSHE has granted full approval to the above named project, for a period of **one year (July 28, 2021- July 27, 2022)**.

All your most recently submitted documents have been approved for use in this study. The study should comply with the international and national scientific and ethical standard guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to MoSHE. Please ensure that you submit biannual progress report to MoSHE once in six months and annual renewal application 30 days prior to the expiry date.

We, therefore, request you as PI and your esteemed organization to ensure the commencement and conduct of the study accordingly and wish for the successful completion of the project.

Sincerely

Daniel Taddesse Wolde (PhD)
Research Ethics Director

Cc

- Office of the State Minister (Sector for Science and Community Service)
- Science and Research Affairs Directoriate General
- Research Ethics Directorate
  MoSHE
- Dr.Fitsum Girma (PI)
  AHRI

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Annex 1.8. AHRI/ ALERT Ethics Committee Approval Letter

8.10. AAERC Approval letter

AHRI/ALERT Ethics Review Committee

Date: February 15, 2021

NO:________

ANNEX 4
Form AF-10-015

AAERC approval letter

Protocol number **PO/46/20**

Investigators: **Fitsum Girma**

Protocol Title: **"African Centre for hrp2/3 Deletion Surveillance (ACHIDES) – Ethiopia"**

Study Site(s): **Ethiopia**

Application Type: Initial ■ Amendment ☐ Renewal ☐

Review Procedure: Full Board ■ Expedited ☐ Secretariat ☐

Review Date: **January 14, 2020** Review Decision: Approved

Final Decision: ■ Approved Approval Date: **February 15, 2021**

Approval period: **February 15, 2021** to **February 14, 2022**

I. Elements approved- 1. Protocol Version No. 2.0 Version Date: February, 2021
2. ICF Version No. 2.0 Version Date: February, 2021

II. Obligations of the Principal Investigator-

1. Should comply with standard international & national scientific and ethical guidelines.
2. All amendments and changes made in protocol and consent form need AAERC approval.
3. SAE should be reported to AAERC within 10 days of the event.
4. End of the study, including manuscripts and thesis works should be reported to the AAERC.

III. Does the protocol need to be reviewed by the National ERC (NRERC)? Yes ■ No ☐

Follow up report expected in:

3 Months ____ 6 Months ____ 9 Months ____ One year ✓

Name:	Hailemichael Getachew	Dr. Getnet Yimer	Abebe Genetu Bayih (PhD)
Signature:			
Date:	15/02/21	15-02-21	17/02/2021
	AAERC Secretary	AAERC Chairperson	AHRI Director General