Molecular Epidemiology of Anti-malarial Drugs Resistance Markers in Clinical *Plasmodium falciparum* Isolates and Infectiousness of Symptomatic Patients to Mosquitoes in East Shewa Zone, Oromia Regional State, Ethiopia

Jifar Hassen

A Dissertation Submitted to

The Department of Applied Biology

School of Applied Natural Science

Presented in Fulfillment of the Requirement for the Degree of Doctor of Philosophy in Biotechnology (Specialization in Health Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

April, 2023

Adama, Ethiopia

Molecular Epidemiology of Anti-Malarial Drugs Resistance Markers in Clinical *Plasmodium Falciparum* Isolates and Infectiousness of Symptomatic Patients to Mosquitoes in East Shewa Zone, Oromia Regional State, Ethiopia

Jifar Hassen

Major Supervisor: Hunduma Dinka (Ph.D., Professor)

Co-supervisor: Lemu Golassa (Ph.D., Associate Professor)

A Dissertation Submitted to

The Department of Applied Biology

School of Applied Natural Science

Presented in Fulfillment of the Requirement for the Degree of Doctor of Philosophy in Biotechnology (Specialization in Health Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

April, 2023

Adama, Ethiopia

DECLARATION

I hereby declare that this Dissertation entitled **"Molecular Epidemiology of Anti-malarial Drugs Resistance Markers in Clinical *Plasmodium falciparum* Isolates and Infectiousness of Symptomatic Patients to Mosquitoes in East Shewa Zone, Oromia Regional State, Ethiopia"** is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other University. All sources of materials used for this thesis have been duly acknowledged through appropriate citations.

_____	_____	_____
Name of student	Signature	Date

RECOMMENDATION

I/we, the supervisor(s) of this dissertation, hereby certify that I/we have read and revised the dissertation entitled **"Molecular Epidemiology of Anti-malarial Drugs Resistance Markers in Clinical *Plasmodium falciparum* Isolates and Infectiousness of Symptomatic Patients to Mosquitoes in East Shewa Zone, Oromia Regional State, Ethiopia"** prepared under my/our guidance by **Jifar Hassen** submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in biotechnology (specialization in health biotechnology).

Hunduma Dinka (Ph.D., Professor)		
Major Supervisor	Signature	Date
Lemu Golassa (Ph.D., Associate Professor)		
Co-supervisor	Signature	Date

APROVAL PAGE

ACKNOLEDGEMENTS

Due to Allah the Almighty and mercy blessings, I could finally complete this dissertation.

First of all, I would like to express my sincerely thanks, to my supervisor Professor Hunduma Dinka from Adama Science and Technology University who has kept on guiding me from the beginning up to the end of my writing up of this dissertation. Under his supervision, I learned a lot from his scientific way of thinking, writing scientific papers, exchange of ideas and issues.

I am deeply indebted to my co-supervisor Dr. Lemu Golassa from Addis Ababa University, Aklilu Lemma Institute of Pathobiology for his invaluable suggestions, guidance and constructive comments throughout this dissertation work. I would like to thank him also for facilitating the whole laboratory work and provision of budget required for the experiments through Human, Heredity and Health Africa (H3Africa) [H3A-18-002] project.

I would like to acknowledge Oromia Education Bureau and Robe Teachers' College for sponsorship and Adama Science and Technology University for giving me the chance to pursue the Ph.D. program.

My appreciation also goes to Adama public health research and referral laboratory and Aklilu Lemma Institute of Pathobiology for provision laboratory set-up and facilities required for membrane feeding and molecular experiments, respectively.

Grateful acknowledgements also goes to Oromia Health Bureau, East Shewa, Adama town and study areas health offices for facilitating conditions to collect data required for this study.

My grateful extends to study participants for their willingness to participate in the study and laboratory technologists who were involved in collecting and examining blood specimens collected from patients attending health facilities.

My special acknowledgement goes to Mr. Andargie Abate, Mr. Adugna Belaw, Ms. Soria Kedir, Mr. Mitiku Bose, Dr. Gezahegn Solomon, Ms. Tewabech Lemma, Ms. Tsehay Orlando, Ms. Yergalem G/Hiwot, Ms. Roza Yosef and Mr. Wessen Sisay who were involved in data collections and laboratory experiments.

I would like to Acknowledge Robe Teachers' College and ASTU staff members and my colleagues Dr. Temesgen File, Mr. Mihrat Yirgu, Mr. Behailu Samuel, Mr. Kenasa Chali, and Ms. Genet Atsebh of the department of Biology for their sharing of ideas and experiences.

My grateful also extends to my relatives: Mr. Awel Muhammed, Ms. Zahara Kurkura, Mr. Anwar Kassim, Mr. Jewar Kassim, Mr. Abdulkerim Kassim and others who stood by my side through provision of moral and materials during this Ph.D. study.

Last but not the least; my acknowledgement is extended to my lovely spouse (Jemila Kurkura) who supports me morally, financially and taking care of our children (Hasenet, Nayad and Rahmet). My grateful extends to my brothers (Muhammed Hassen and Kedir Hassen) and sisters (Duri Hassen, Yeba Hassen and Jeware Hassen) and all others who stood by my side during this study.

TABLE OF CONTENTS

CONTENTS **PAGE**

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

ACTs	Artemisinin-based combination therapies
AL	Artemether-lumefantrine
API	Annual parasite incidence
APoI	*Artherobacter protophormiae I*
AQ	Amodiaquine
ART	Artemisinin
AS	Artesunate
ATSB	Attractive toxic sugar baits
CQ	Chloroquine
CQR	Chloroquine resistance
CQS	Chloroquine sensitive
DBS	Dried blood spot
DDT	Dichloro-Diphenyl Tiricholoroethane
DHA	Dihydro artemisinin
DHFR	Dihydrofolate reductase
DHPS	Dihydropetroate synthase
DMFAs	Direct membrane feeding assays
DNA	Deoxyribonucleic acid
DV	Digestive vacuole
EPHI	Ethiopian Public Health Institute
G6PD	Glucose-6-phosphate dehydrogenase
HF	Halofantrine
IRS	Indoor residual spraying
ITNs	Insecticide treated nets
LAMP	Loop-mediated isothermal amplification
LF	Lumefantrine
MOH	Ministry of Health
MQ	Mefloquine
NASBA	Nucleic acid sequence based amplification

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
*Pf*ATPase6	*Plasmodium falciparum* adenosine triphosphatase-6
Pfcrt	*Plasmodium falciparum chloroquine transporter resistance*
*Pf*DHFR	*Plasmodium falciparum* dehydrofolate reductase
*Pf*HRP	*Plasmodium falciparum* histidine-rich protein
Pfmdr1	*Plasmodium falciparum multi-drug resistance1*
*Pf*MGET	*Plasmodium falciparum* male gametocyte-enriched transcript
*Pf*PI3K	*Plasmodium falciparum* phosphatidylinositol-3-kinase
QN	Quinine
RBCs	Red blood cells
RDTs	Rapid diagnostic tests
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
SFA	Skin feeding assay
SMFA	Standard membrane feeding assay
SNPs	Single nucleotide polymorphisms
SP	Sulfadoxine-pyrimethamine
SSA	sub-Saharan Africa
TRIs	Transmission reducing interventions
UV	Ultra-violet
WBCs	White blood cells
WHO	World Health Organization

ABSTRACT

Surveillance of antimalarial drug resistance markers and membrane feeding experiments are among the efforts of controlling and/or eliminating malaria. Plasmodium falciparum resistance to every class of anti-malarial drugs is a major challenge in efforts to control and/or eliminate malaria worldwide. Due to widespread chloroquine (CQ) resistant P. falciparum Ethiopia changed its treatment policy from CQ to sulfadoxine-pyrimethamine (SP) in 1998. However, resistance to SP developed shortly and in 2004 artemether–lumefantrine (AL, Coartem®) was recommended as a first-line treatment for the uncomplicated falciparum malaria. Data on the prevalence of CQ resistance markers after more than two decades of its removal is important to map the selection pressure behind the targets codons of interest. Understanding human malaria infectious reservoir is important for malaria elimination initiatives. It is poorly studied in low malaria transmission settings. As a consequence, data of onward transmission potential of malaria infected patients to mosquitoes are scant in Ethiopia. The present study was conducted to determine the prevalence of mutations in Pfcrt K76T and Pfmdr1 N86Y codons and to assess infectiousness of symptomatic Plasmodium infected patients to colonies of Anopheles arabiensis mosquitoes in East Shewa Zone, Oromia Regional State, Ethiopia. Finger-prick blood samples were collected on 3MM Whatman ® filter papers from a total of 121 microscopically confirmed P. falciparum infected patients. Parasite DNA was extracted by Chelex-100 method from dried blood spot (DBS). Genomic DNA was used to amplify both codons by nested PCR. Nested PCR products were subjected to APoI endonuclease digestion to determine mutations at codons 76 and 86 of Pfcrt and Pfmdr1 genes, respectively. Direct membrane feeding assays (DMFAs) were conducted on 63 patients infected with P. falciparum, P. vivax and mixed infections. Mosquitoes' mid-guts and salivary glands were dissected to evaluate oocyst and sprozoite infection rates, respectively. Out of 83 P. falciparum isolates successfully genotyped for Pfcrt K76T, 91.6% harbored 76T mutant genotypes. The prevalence of Pfcrt 76T was 95.7%, 92.5% and 84.5% in Adama, Metehara and Olenchiti study areas, respectively. However, the prevalence of this mutation in the three study areas showed no statistical significance difference ($\chi 2 = 1.895$; $P = 0.388$). On the other hand, among 80 P. falciparum samples successfully amplified for Pfmdr1 N86Y all carried Pfmdr1 N86 wild-type genotypes. Out of 63 DMFAs, 68.3% resulted in at least one infected mosquito with a total infection rate of 21.6% of mosquitoes infected with 1-285 oocysts per mid-gut. Among 28 randomly selected assays with oocyst infected mosquitoes, 60.7% had sprozoites in the salivary glands of mosquitoes. *Both mosquitoes (oocysts) and sporozoite infection rates demonstrated positive correlation with parasitaemia and gametocytaemia. Demonstrating oocyst infection in the mosquitoes might predict infectiousness of mosquitoes, although not all infected mosquitoes became infectious mosquitoes. The greater proportions of P. falciparum clinical isolates carrying mutant 76T genotypes may be due to indirect CQ pressure following partial withdrawal of CQ in the country. The current fixation of Pfmdr1 N86 allele may be favored by the use of AL for the treatment of uncomplicated falciparum malaria. Study by DMFA showed high infectiousness of symptomatic patients. Patients with symptomatic P. vivax infections were found to be more infectious to mosquitoes than patients with P. falciparum symptomatic infections. The current high prevalence of 76T mutant allele (CQ-resistant) and N86 wild-type allele (Al less susceptible) showed that continues monitoring implementation of CQ and AL should take place in order to contain the spread of antimalarial drug resistance P. falciparum. Further DMFA studies on human and mosquito determinant factors including both symptomatic and asymptomatic malaria infections with larger sample*

size should be carried out using microscopy and molecular techniques in order to guide malaria control and elimination efforts.

Keywords: *Anti-malarial drug resistance, Direct membrane feeding assay, oocysts, Pfcrt, Pfmdr1, Plasmodium falciparum, Plasmodium vivax, sporozoites*

CHAPTER 1

1. INTRODUCTION

1.1. Background

1.1.1. Global Malaria Situation

Historically, the word malaria was derived from “mala aria” (Italian word) which means “bad air” (Service & Townson, 2002). Ancient populations believed that the disease was associated with swampy, marshy areas where the air smelled bad. However, this is wrong belief and it is a protozoan disease caused by the genus *Plasmodium* transmitted by the bite of infected female *Anopheles* mosquitoes. It is one of the leading causes of morbidity and mortality (Benelli & Beier, 2017).

Malaria is among the most infectious diseases and its burden persists particularly in sub-Saharan African (SSA) countries. According to World Health Organization (WHO) malaria report of 2022, malaria cases increased from 241 million in 2020 to 247 million in 2021 but deaths due to malaria slightly decreased from 627 thousand to 619 thousand in 2021. WHO African region alone, contributes to 234 million (95%) and 593 thousands (96%) cases and deaths of malaria, respectively. WHO Southeast Asian and Eastern Mediterranean regions accounted for the rest 5% of malaria cases. Children under five years of age are the most risk group, accounting approximately for 76% of all malaria deaths globally in the same year (WHO, 2022). Twenty- nine countries contributed to 96% of global malaria cases in 2021. Four countries in SSA accounted for nearly half of all malaria cases globally: Nigeria (26.6%), the Democratic Republic of the Congo (12.3%), Uganda (5.1%), Mozambique (4.1%), (WHO, 2022).

Between 2000 and 2015 malaria burden was significantly declined (Bhatt *et al*., 2015). Malaria control interventions such as insecticide treated nets (ITNs) and artemisinin-based combination therapies (ACTs) played significant role for such achievement. Based on the progresses made so far, globally many malaria endemic countries are striving to eliminate and eradicate malaria by 2030 and 2040, respectively (Killeen *et al*., 2017). Twenty countries have eliminated malaria transmission and reported zero cases for at least one year between 2000 and 2017 (Dabaro *et al*., 2021). Iran and Malaysia reported zero indigenous cases for the fourth

consecutive year while Belize and Cape Verde reported zero indigenous cases for the third time in 2021. China and El Salvador received certificate of malaria free from WHO in 2021 (WHO, 2022).

However, since 2016 the progress of reducing worldwide malaria has stalled. The current 2022 WHO malaria report is the noticeable example. Estimated number of cases in 2021 is slightly higher than it was in 2000 (247 million versus 245 million) (WHO, 2022). Therefore, this calls for more efforts to control and eliminate malaria.

1.1.2. Malaria Situation in Ethiopia

Malaria is one of the major public health and socioeconomic problems in Ethiopia, despite its dramatic reduction in the last two decades. Apart from its morbidity and mortality, it caused persistent socioeconomic impacts specifically to 80% of the rural community. Malaria alone accounts for loss of 30% of all disability adjusted life years (Ministry of Health [MOH], 2020). Nearly, 68% of its landmass (below 2000m) has suitable conditions for breeding of *Anopheles* mosquitoes and hence 60% (54 million) of the population at risk of malaria (MOH, 2014; Gari & Lindtjørn, 2018).

In the most parts of the country, malaria transmission is seasonal and unstable: major transmission is from September to mid-December following main rainy season (June - August) while minor transmission is during April to May (MOH, 2020). Malaria transmission in most part of the country overlaps with the seasons of planting and harvesting (MOH, 2017). It is often characterized by frequent focal or sometimes large scale epidemics (Gari & Lindtjørn, 2018; MOH, 2020). Since 1958, major malaria epidemics tended to occur in 5-8 years intervals; however since 2003 there has not been major malaria epidemic except smaller-scale outbreaks and seasonal case build-ups (MOH, 2020). Infrequently, higher rainfall, temperature and humidity could be the driving forces for the occurrence of such epidemics (MOH, 2020). In addition to climatic factors, altitude influences malaria transmission intensity. As the elevation increases temperature decreases and vice versa that in turn influences the density of *Anopheles* mosquitoes and hence impact transmission dynamics. More malaria cases usually occurred at lower altitude compared to higher altitude (Dabaro *et al*., 2021).

In Ethiopia *P. falciparum* and *P. vivax* co-exist in the proportion of approximately 60% and 40%, respectively (Alelign & Dejene, 2016). This relative proportion varies with time, space and altitude. For example, from malaria sentinel sites 80.1% of *P. falciparum* and 33.2% of *P. vivax* rates were reported in lowlands and highlands, respectively (MOH, 2020). Malaria cases due to *P. malariae* and *P. ovale* are very rare in Ethiopia (MOH, 2012; Alemu *et al*., 2013).

Anopheles arabiensis, a member of *An. gambiae senso lato* (*s.l.*) complex, is the main vector of malaria in Ethiopia, while *An. pharoensis*, *An. funestus* and *An. nili* are secondary vectors in some areas (Taye *et al*., 2006; Massebo *et al*., 2013). *Anopheles stephensi*, an Asian malaria vector, which was found in Middle East, Indian subcontinent, China, Africa (Djibouti), is a new malaria vector detected for the first time in Kebridehar (Somali Region of Ethiopia) in 2016 (Carter *et al*., 2018) and it is widely distributed in the eastern parts of Ethiopia (Balkew *et al*., 2020). It is competent vector for both *P. falciparum* and *P. vivax* malaria parasites in urban and rural settings (Sinka *et al*., 2020). Infectivity study through membrane feeding experiment done in Ethiopia by Tadesse *et al*. (2021) confirmed the susceptibility of *An. stephensi* for both *Plasmodia* species.

In the past decade, Ethiopia has implemented key malaria interventions throughout the country. Indoor residual spraying (IRS) using Dichloro-Diphenyl-Tiricholoroethane (DDT) was introduced first in Ethiopia in 1959 as a means of global malaria eradication campaign and then different chemical insecticides such as pyrethroids have been used for malaria control (Gari & Lindtjørn, 2018; Taffese *et al*., 2018). ITNs were introduced as additional malaria intervention after 38 years (1997) of implementation of IRS (Gari & Lindtjørn, 2018). Malaria cases were treated with CQ until 1998. Due to widespread of CQ resistant *Plasmodium* parasites, it was replaced with sulfadoxine-pyrimethamine (SP) in 1998 for the treatment of uncomplicated falciparum malaria; but CQ persists for the treatment of vivax malaria (MOH, 2017). Therapeutic efficacy of SP was dropped soon because parasites developed resistance and this initiated the country to replace SP with artemether-lumefantrine (AL, Coartem®) as a first- line drug for the treatment of uncomplicated falciparum malaria. Gametocytocidal single low dose (0.25mg per kg) primaquine (PQ) is given to falciparum and vivax malaria cases as per the WHO recommendations (WHO, 2015; MOH, 2017).

According to recent WHO and Ethiopian Federal MOH reports (MOH, 2020; WHO, 2020), there is a significant decline in morbidity and mortality, putting Ethiopia among the few African countries on track to meet the global 2020 milestone of cutting incidence by 40% or more. These initiations enabled the Ethiopian national malaria control program to stratify the country's (1,046 districts) malaria transmission into five strata based on annual parasite incidence (API), attitude and expert opinions (MOH, 2020). Accordingly, the five strata are: malaria free (API ~0 cases/1000 population/year), very low (API > 0 and ≤ 5), low (API > 5 and < 10), moderate (API ≥ 10 and < 50) and high (API ≥ 50) as a preparation to embark on nationwide malaria elimination.

In the last decade large scale implementation and sustained coverage of mosquito control and anti-malarial interventions enable the country to develop national malaria strategic plan to eliminate malaria by 2030 which is aligned with WHO Global Technical Strategy for malaria (2016-2030) (MOH, 2020). Many malaria districts have reduced annual malaria incidence that exceed the criteria of WHO pre-elimination phase (API > 0 and < 5) (WHO, 2008a) and the country is currently moving toward the pre-elimination phase (Bugssa & Tedla, 2020).

1.1.3. Anti-malarial Drug Resistance Markers in *Plasmodium falciparum*

Plasmodium falciparum has developed resistance to a number of anti-malarial drugs. Resistance to anti-malarial drugs is due to single nucleotide polymorphisms (SNPs) or duplication in target and/ or transporter genes of *P. falciparum.* The commonest SNPs include: *P. falciparum chloroquine resistance transporter* (*Pfcrt*) gene located on chromosome 7 is associated with Chloroquine (CQ) resistance (Fidock *et al*., 2000); *P. falciparum* multidrug resistance 1 (*Pfmdr1*) gene located on chromosome 5 is associated with tolerance or susceptibility to CQ, mefloquine (MQ), lumefantrine (LF) and amodiaquine (AQ) (Sanchez *et al*., 2010); dihydropetroate synthase (dhps) and dihydrofolate reductase (dhfr) genes located on chromosomes 8 and 4, respectively are associated with sulfadoxine and pyrimethamine resistance, respectively (Saifi *et al*., 2013) and Kelch 13 gene located on chromosome 13 is associated with ART resistance (Ariey *et al*., 2014). Increased *Pfmdr1* gene copy number is also attributed to resistance to halofantrine (HF), MQ, ART and LF (Ibraheem *et al*., 2014; Okell *et al*., 2018).

1.1.4. Mosquito Infectiousness Studies for Malaria Control

Transmission of malaria from man to mosquitoes indicates that humans are infectious reservoirs of malaria. For epidemiological studies this transmission can be assessed by mosquito feeding experiments (Bousema *et al*., 2012; Tadesse *et al*., 2018; Timinao *et al*., 2021). In such experiments wild or laboratory (colony) reared *Anopheles* mosquitoes are allowed to feed directly on the skin of malaria infected patients or in blood sample provided through a membrane and then the mosquitoes are dissected for the assessment of oocysts (in mid-gut) and/or sporozoites (in salivary glands) (Bousema and Drakeley, 2011; Bousema *et al*., 2012).

One of the strategies of malaria control is to focus on reduction of gametocyte transmission from human reservoirs or to interfere with sporozoite development in malaria vectors (Bousema *et al*., 2013). Therefore, mosquito feeding experiments are valuable tools in evaluating the efficacy of malaria transmission reducing interventions (TRIs) that target gametocytes and sporozoite stages of the malaria parasites (Bousema *et al*., 2013; Miura *et al*., 2016; Stone *et al*., 2017). At the same time membrane feeding experiments are also used to measure who contributes the most to the infectious reservoir from the population (Bousema and Drakeley, 2017; Tadesse *et al*., 2018). This helps for optimal use of TRIs to reduce the onward transmission of malaria to mosquitoes.

1.2. Statement of the Problem

Anti-malarial drug resistance is a big challenge in the effort to control and/or eliminate malaria globally. Particularly, *P. falciparum* has developed resistance to every classes of anti-malarial drugs currently available including ART (WHO, 2017; Roux *et al*., 2021). Analysis of molecular markers associated with drug resistance is the commonly employed strategy in the surveillance of anti-malarial drug resistance in *Plasmodium* parasites (Nsanzabana *et al*., 2018).

Due to the emergence and widespread of anti-malarial resistant *P. falciparum* to mono-therapy, since 2001 WHO adopted ACTs for the treatment of uncomplicated falciparum malaria in malaria endemic countries (WHO, 2008b). This switch to ACTs was followed in some countries by the fast rise of *P. falciparum* strains possessing wild-type markers such as *Pfcrt* K76 and *Pfmdr1* N86, susceptible to CQ in the hope of re-introduction of CQ as mono or

combination therapy in the future (Kublin *et al*., 2003; Mohammed *et al*., 2013; Mulenga *et al*., 2021).

After introduction of AL (ACT-based) in Ethiopia, previous studies indicated high prevalence and in some areas fixation of *Pfcrt* lysine (K) 76 therionine (T) (K76T) mutation and *Pfmdr1* asparagine (N) thyroxine (Y) (N86Y) wild-type *P. falciparum* parasites (Golassa *et al*., 2015; Lo *et al*., 2017; Hailemeskel *et al*., 2019). Only one study showed high prevalence recovery of CQ sensitive (*Pfcrt* K76) *P. falciparum* parasites (Mekonnen *et al*., 2014). However, the prevalence of these codons was not documented throughout the country.

Human infectious reservoir of malaria is the transmission of malaria parasites from humans to mosquitoes. This onward transmission of *Plasmodia* to mosquitoes is a less studied field of research. Most of the field studies employed skin feeding assay (SFA) and/or direct membrane feeding assay (DMFA) (Bousema *et al*., 2012; Goncalves *et al*., 2017; Tadesse *et al*., 2018).

Mosquito infectiousness studies showed that symptomatic *P. vivax* infections are more infectious to mosquitoes than *P. falciparum* infections (Tadesse *et al*., 2018; Timinao *et al*., 2021) due to rapid mature gametocytes maturations in *P. vivax* (Bantuchai *et al*., 2021). In highly endemic malaria area, more data for infectiousness of asymptomatic *P. falciparum* are available (Queraugo *et al*., 2016; Goncalves *et al*., 2017) suggesting that asymptomatic infections carry infective gametocytes for long period. However, studies from low-transmission settings such as Cambodia (Lin *et al*., 2016), Ethiopia (Tadesse *et al*., 2018) and The Gambia (Ahmad *et al*., 2021) symptomatic falciparum infections with gametocytes detected by microscopy are becoming important contributors of mosquito infections.

Mosquito infectiousness studies usually conducted in high malaria transmission areas (Goncalves *et al*., 2017). Human infectious reservoir was less studied in low transmission areas (Ahmad *et al*., 2021). As malaria transmission declines in Ethiopia (MOH, 2020), the proportion of infections with low parasite densities may increase. These could serve as an important source of onward transmission of malaria parasites to mosquitoes as a consequence malaria transmission is continued in the area. As far as our knowledge is concerned there was only one study conducted in Ethiopia to measure the relative contribution of malaria transmission from symptomatic and asymptomatic infections to mosquitoes from low malaria endemic setting (Tadesse *et al*., 2018). Data obtained help to control and eliminate malaria,

further studies that assess infectious reservoir in this low transmission area has paramount importance.

Therefore, this study was aimed to investigate the prevalence of *Pfcrt* K76T and *Pfmdr1* N86Y codons in *P. falciparum* isolates and to determine infectiousness of symptomatic patients to *An. arabiensis* mosquitoes in East Shewa zone, Oromia Regional State, Ethiopia.

1.3. Significance of the Study

Chemotherapy for long period persists as one of the methods to fight against falciparum malaria; however extensive utilization of anti-malarial drugs has exerted selection pressure on *P. falciparum* and paved the way for the spread of resistant parasites which became the main obstacle to the hope of elimination of malaria (Murmu *et al*., 2021).

Withdrawal of anti-malarial drugs hinders the spread of resistance parasites and facilitates the re-emergence of susceptible parasites for anti-malarial drug of interest (Laufer and Plowe, 2004). Surveillance of molecular markers may guide for re-introduction of anti-malarial drug whose efficacy is returned either as monotherapy or combination therapy. No recent information about anti-malarial drug resistance is available in the study area; thus, the findings of the study can serve as a baseline information for those scholars who want to undertake further investigation in the study area. The findings may also help government health officials to follow the implementation of the current front line anti-malarial drugs (CQ and AL) used for the treatment of uncomplicated malaria.

Reduction in transmission is the main component of global efforts to control and eliminate malaria. It is highly important in areas approaching pre-elimination and elimination of malaria (Churcher *et al*., 2013). Malaria elimination requires detail understanding of human infectious reservoir to biting mosquitoes (Bousema *et al*., 2012; Gonçalves *et al*., 2017). Identifying individuals who contribute most to the reservoir of infections enables for implementing optimal malaria control interventions. Relative contributions of different groups in field settings are mostly determined by DMFAs (Bousema *et al*., 2012; Tadesse *et al*., 2018). In this study DMFA was conducted to determine infectiousness of malaria symptomatic patients to laboratory reared *An. arabiensis* mosquitoes. The findings of the current study will help government health offices and partners to develop plan that will help to control malaria. The

findings may also important for designing future evaluation of TRIs that will support malaria control and/or elimination strategeies.

1.4. Objectives of the Study

1.4.1. General Objective

- The study aimed to assess prevalence of antimalarial drug resistance markers in *P. falciparum* and transmission of malaria from human to *An. arabiensis* mosquitoes in East Shewa zone, Oromia Regional State, Ethiopia

1.4.2. Specific Objectives

- To determine the prevalence of *Pfcrt* K76T and *Pfmdr1* N86Y mutations among *P. falciparum* infected patients attending health facilities in East Shewa zone, Oromia Regional State, Ethiopia
- To explore infectiousness of symptomatic patients to *An. arabiensis* in East Shewa zone, Oromia Regional State, Ethiopia

1.5. Limitations of the Study

Due to financial and logistic constraints small sample sizes were considered both for anti-malarial drug resistance and mosquito infectiousness studies. Small sample sizes may have impact to draw firm conclusion. The study lacks data of therapeutic efficacy of AL for the treatment of uncomplicated falciparum malaria to associate the outcomes with current high prevalence of *Pfmdr1* N86 allele. Molecular techniques were not employed to quantify gametocyte and oocyst densities for mosquito infectivity. Lack of molecular techniques hindered quantifications of gametocytes that were undetected by microscopy. In low density infections it was difficult to differentiate oocyst from artifacts by using only microscopy. Additionally, confounding factors which may influence the outcome of mosquito infectivity such as immunity, genetics, nutrition, and health status of the study participants were not considered.

CHAPTER 2

2. LITERATURE REVIEW

2.1. The Malaria Parasite

Malaria parasite belongs to the phylum Apicomplexa, the order of Haemosporida, the family of Plasmodidae and genus *Plasmodium.* Five *Plasmodium* species are known to cause malaria in humans: *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* (WHO, 2015). From clinical perspectives, *P. falciparum* and *P. vivax* cause severe disease to humans (WHO, 2022). *Plasmodium falciparum* is the most prevalent and major causes of malaria cases and deaths in SSA whereas, *P. vivax* is the most prevalent in Southeast Asia and America regions (WHO, 2022). Compared to *P. falciparum*, *P. vivax* has a wider geographic distribution because it can develop in the *Anopheles* mosquito vectors at lower temperatures and it can survive in higher altitudes and cooler climates (Zheng & Cheng, 2017; MOH, 2020). More than 80% of *P. vivax* malaria cases are estimated to occur in three countries: Ethiopia, India and Pakistan (Zheng & Cheng, 2017). Although *P. malariae* may occur in all malarious areas, its prevalence is generally low. In tropical Africa, *P. falciparum* and *P. malariae* co-infection is sometimes encountered. *Plasmodium ovale* is widespread principally in tropical Africa; currently two distinct subspecies *P. ovale curtisi* and *P. ovale wallikeri* of *P. ovale* have been identified (Autino *et al*., 2012; Alemu *et al*., 2013). *Plasmodium knowlesi* which causes malaria to long-tailed and pig-tailed macaques monkey has also been found to infect humans in certain forested areas of Southeast Asia (Autino *et al*., 2012). Transmission of *P. knowlesi* to humans is possible when *Anopheles* mosquito previously infected with the monkey malaria parasite bites humans.

2.2. *Anopheles* Vectors

Malaria transmission occurs by the bite of female *Anopheles* mosquitoes when feed on a blood of an infected individuals for the purpose of reproductive process. There are more than 500 *Anopheles* species that are recognized globally, whereas 70 species are able to transmit *Plasmodium* to humans. Forty-one *Anopheles* species are defined as the most important malaria vectors globally, causing the majority of human malaria cases (Autino *et al*., 2012). *Anopheles gambiae s.l. complex* compose of morphologically indistinguishable sibling species

are the most effective and efficient malaria vector with highest entomological inoculation rates and responsible for the highest malaria prevalence globally (Autino *et al*., 2012; White *et al*., 2013).

2.3. Life Cycle of Malaria Parasites

The life cycle of malaria parasites that infect humans is a complex developmental stage involving human host and Anopheline mosquito vector (White *et al*., 2013). The life cycle involves: mosquito, liver and blood stages (Figure 1). Female *Anopheles* mosquitoes receive *Plasmodium* parasites from humans when feed on infected blood meal and transmit to healthy individual during the next blood meal. The infective stages, sporozoites, which exist in the mosquito salivary glands are inoculated into the skin together with saliva. Some sporozoites through circulation or lymphatic system travel to the liver where they invade hepatocytes. Within hepatocytes successful sporozoites multiply by a process called schizogony to form 10,000-30,000 daughter merozoites within 5.5-8 days (White *et al*., 2013). Hepatocytes containing schizonts bursts and liberates merozoites that invade red blood cells (RBCs). In the cases of *P. vivax* and *P. ovale* some sporozoites form dormant stages or hypenozoites that can persist in the liver for several weeks or years later activate, enter the bloodstream and cause clinical disease (White, 2011). Within RBCs the parasites pass through different developmental stages (ring stages, trophozoites and schizonts) and finally the RBCs burst and release approximately 16-32 merozoites (Andrade *et al*., 2020). The newly released merozoites re-infect RBCs and the parasite completes the asexual cycle in the blood roughly at every 48h for *P. falciparum, P. vivax* and *P. ovale*, 24h for *P. knowlesi* and 72h for *P. malariae* (Sato, 2021). The pathology of malaria infection and its associated clinical manifestations are mainly attributed to these blood stages of the parasites. Symptoms usually manifest within 6-8 days after they are released from the liver (White *et al*., 2013). During the blood stage, some of the merozoites develop into longer lived sexual forms (gametocytes). Male and female gametocytes are then taken up by *Anopheles* mosquitoes during the next blood meal. Based on unique environmental conditions in the mid-gut of mosquito male and female gametocytes differentiate into eight flagellated microgamete and a single macrogamete, respectively (Meibalan & Marti, 2017). Sexual reproduction takes place and a zygote (the only diploid stage) is formed that will develop via meiosis into an elongated form, ookinete. The ookinete crosses the mid-gut epithelium and turns into spherical oocyst (the longest developmental

stage in the mosquito) under the basal lamina of mid-gut of the epithelium (Musiime *et al*., 2019). Within oocyst repeated rounds of cell division takes place and several haploid sporozoites are formed and released upon oocyst burst which will migrate via haemolymph to the salivary glands of the mosquito. From the salivary glands sporozoites are ready to infect human host when bitten by the mosquitoes. For human infecting *Plasmodium* species the development from gametocytes to mature sporozoites takes approximately 10-18 days (Sato, 2021).

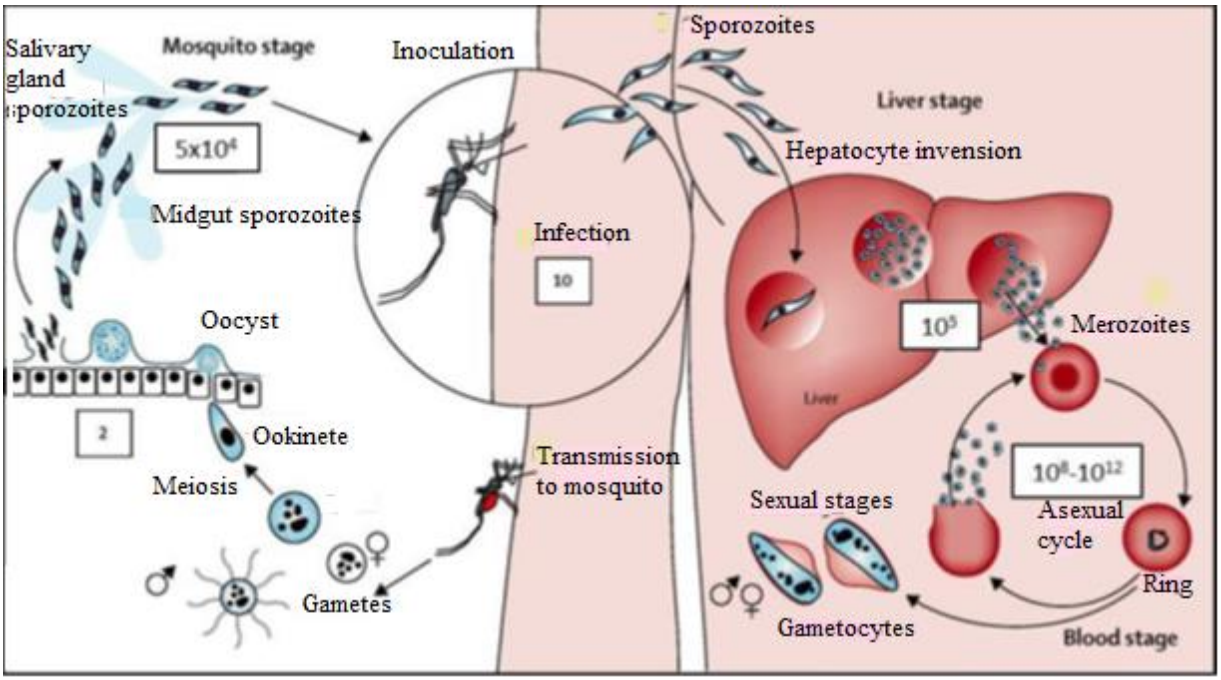


Figure 1. Life cycle of malaria parasites in the human body and anopheline mosquito. Estimated number of parasite stages is indicated in the boxes. Adapted from White *et al*. (2013).

2.4. Clinical Features of Malaria

In endemic areas malaria most often is characterized by fever (cardinal feature of malaria). Symptoms of malaria usually manifest 10-15 days post mosquito bite. Clinical malaria is categorized as uncomplicated and severe (life-threating) malaria (White *et al*., 2013).

2.4.1. Uncomplicated Malaria

Initial symptoms of malaria are non-specific and difficult to distinguish from other viral or bacterial diseases. These symptoms include headache, fatigue, muscle aches, abdominal

discomfort, nausea, vomiting which usually followed by irregular fever that occurs every 24, 48 or 72 hours depending on *Plasmodium* species (Sato, 2021). Most patients with uncomplicated malaria may have mild anaemia and a palpable spleen. Enlargement of liver may occur in young children, while jaundice most likely occurs in adults. If uncomplicated malaria left untreated it can lead to severe malaria and possibly fatal outcome (White *et al*., 2013).

2.4.2. Severe Malaria

Severe malaria is usually associated with *P. falciparum* which depends on age. Manifestations of severe malaria include severe anaemia, hyperparasitaemia, cerebral malaria, hypoglycaemia, pulmonary oedema, jaundice, acute kidney injury, metabolic acidiosis and acute respiratory distress. Severe anaemia and hypoglycaemia are often occurred in children while acute pulmonary oedema and jaundice are usually occurred in adults. Acidiosis and coma due to cerebral malaria occur in all age groups (White *et al*., 2013).

2.5. Malaria Diagnoses

Early and accurate diagnosis of malaria is essential for case management and malaria control programs. This kind of diagnosis is also essential to prevent further progression and lowers severity of the disease ((WHO, 2010a). The two approaches of malaria diagnoses are clinical and test-based methods (Lopez *et al*., 2022). Each approach has its own strengths and weaknesses.

2.5.1. Clinical Diagnosis

Clinical diagnosis, which is the presumptive diagnosis of malaria, is the commonest experience of diagnosing malaria as it is very cheap and rapid. It is based on the signs and symptoms those patients manifest during examination (Tangpukdee *et al*., 2009). Although clinical diagnosis is imprecise, it is usually employed for therapeutic care of febrile patients in malaria endemic settings where laboratory based diagnosing is inaccessible particularly in resource scare settings. Overlapping of malaria symptoms with other diseases like typhoid fever, respiratory tract and viral infections impair the specificity of clinical diagnosis and encourages indiscriminate use of anti-malarial drugs in malaria endemic settings (Lopez *et al*. 2022). Incorrect use of anti-malarial drugs also leads to the occurrence of drug resistant

malaria parasites (Khan *et al*., 2020). Level of malaria endemicity, malaria season and age also make clinical diagnosis imprecise (Mwangi *et al*., 2005).

2.5.2. Microscopic Diagnosis

Microscopic diagnosis of *Plasmodium* species is considered as the gold standard for laboratory diagnosis in endemic countries (WHO, 2015). It is the most widely used malaria diagnosing tool all around the world due to its simplicity, low cost, capacity to detect parasites, discrimination of infecting species and estimating parasite concentration. However, microscopy is time consuming, labour intensive, requires well trained personnel, quality reagents and its sensitivity is low particularly at low parasite densities. Although well trained microscopist can detect as low as 5 parasites/µl, average microscopists can detect only up to 50-100 parasites/µl. This can result in underestimating malaria infection rates (reporting false negatives), particularly in individuals with low parasitaemia and asymptomatic cases (Tangpukdee *et al*., 2009; Golassa *et al*., 2015). Such sub-microscopic and asymptomatic individuals with low parasitaemia remain undiagnosed and untreated and become the source of infection in the community (Zheng & Cheng, 2017). Microscopy is also unavailable in rural areas where electricity is problematic.

2.5.3. Rapid Diagnostic Tests

In order to overcome limitations encountered by microscopy, WHO introduced, simple, quick, accurate and cost effective diagnostic tools called RDTs (Tangpukdee *et al*., 2009). These tests greatly enhanced the quality of malaria diagnosis, especially in peripheral health centers/health posts where microscopy would be unavailable. Since 1990s malaria RDTs became available and there are more than 200 brands of RDTs approved and deployed in the field settings (Nyataya *et al*., 2020).

Different types of RDTs use various types of antibodies or combination of antibodies to detect *Plasmodium* antigens. Some antibodies aim to detect a single species while others are pan-malarial, aiming to detect all types of *Plasmodium*. Accordingly, many currently available RDTs can detect *Plasmodium* parasite using one or more target antigens: *Plasmodium falciparum* histidine-rich proteins-2 and/or-3 (*Pf*HRP-2/3), lactate dehydrogenase and aldolase (Tangpukdee *et al*., 2009; Nyataya *et al*., 2020). The majority of commercial RDTs detect HRP-2 (a water-soluble antigen), which is expressed only by *P. falciparum* and this test is

used for specific diagnosis of falciparum malaria. Lactate dehydrogenase and aldolase are detected in non-HRP-2 based tests and commonly used as pan-specific tests for *Plasmodium* parasites, but tend to yield lower diagnostic accuracy in commercially available RDTs (Han, 2013; Nyataya *et al*., 2020).

The limit of detection of most available RDTs is 200 parasites/µl, sensitivity declines for lower parasite densities (Han, 2013). False negativity may also occur due to deletion of protein sequences (*Pf*HRP-2 and/or *Pf*HRP-3) in circulating parasite strains which might negatively impact the success of RDT-based diagnosis and pose threat to the progress made in the fight against malaria. Parasites that lack *Pfhrp-2/3* gene cannot express *Pf*HRP2/3 proteins and cannot be detected by *Pf*HRP-2/3 based RDTs (WHO, 2022). Currently, high prevalence of parasites lacking *Pf*HRP-2 and *Pf*HRP-3 were reported from different countries (Berhane *et al*., 2018; Alemayehu *et al*., 2021).

2.5.4. Nucleic Acid-based Amplification Techniques

Nucleic acid-based amplification techniques are the most accurate and sensitive methods of identifying low levels of parasitaemia, parasite species and mixed infections. They are particularly useful in detecting asymptomatic and sub-patent infections missed by microscopy and RDTs (Golassa *et al*., 2015). These techniques include PCR and isothermal-based methods (Zheng & Cheng, 2017).

2.5.4.1. PCR-based Methods

PCR-based techniques are recently developed in the molecular malaria diagnosing laboratories. They are particularly important for the detections of malaria cases with low or mixed infections or drug resistant parasites. PCR-based techniques can detect parasitaemia level as low as 1-5 parasites/µl while some of the throughput techniques have detection limit of 0.002 parasites/µl (Kamau *et al*., 2011). Nested PCR is highly sensitive and specific in detecting *Plasmodium* parasites. It is a reliable molecular technique for detection of low density infections routinely missed by microscopy and RDTs (Golassa *et al*., 2015). Real-time PCR avoids multiple amplification reactions required by nested PCR to discriminate *Plasmodium* species. It allows simultaneous identification of *Plasmodium* parasites in a single reaction tube (Genc *et al*., 2010; Zheng & Cheng, 2017). Real-time-PCR assays improved sensitivity and specificity of detection over other PCR techniques. For example, Genc *et al.*

(2010) have reported that the sensitivity of real-time is 100% over the sensitivity of nested PCR. Another PCR technique is a reverse transcriptase-PCR (RT-PCR) which is used to detect RNA transcripts. RT-PCR assay is suitable for detection of low parasite levels in patients with early stage malaria and/or submicroscopic infections. Quantitative RT-PCR is an ideal test for detection and quantification of submicroscopic gametocytes otherwise undetected by microscopy. The quantitative RT-PCR assay developed by Wang *et al*. (2020), for example, able to accurately quantify *Pfs25* of female gametocyte and *P*. *falciparum* male gametocyte-enriched transcript (*PfMGET)* of male gametocyte messenger RNAs at limit of detection of 0.0039/µl and 0.0269/µl, respectively. Although PCR-based techniques display high sensitivity and specificity, they require high standard laboratory set-ups, expensive thermocyclers and reagents and qualified man-power which are unavailable in malaria endemic areas (Han, 2013).

2.5.4.2. Isothermal Methods of Amplification

2.5.4.2.1. Nucleic Acid Sequence-Based Amplification

Nucleic acid sequence-based amplification (NASBA) is a sensitive, transcription based amplification system of nucleic acids using enzymes (Fakruddin *et al*., 2012). Unlike-PCR techniques, it does not require thermocycler because the reaction can be conducted at an isothermal condition (usually at 41^{o}C) producing more than 10^{8}-fold amplification of RNA sequence of interest (Mbanefo & Kumar, 2020). NASBA is highly a valuable tool for quantification of RNA and can detect gametocyte densities as low as 0.02-0.1/µl (Schneider *et al*., 2006). Like RT-PCR, NASBA can also be used to determine the dynamics of human infectious reservoir of malaria by quantifying *Pfs25* messenger RNA (Ouédraogo *et al*., 2016). Although NASBA has high sensitivity and specificity, unstable and thermolabile natures of RNA and enzymes, respectively are some of its limitations (Fakruddin *et al*., 2012).

2.5.4.2.2. Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is an isothermal molecular method that uses deoxyribonucleic acid (DNA) polymerase from *Bacillus stearothermophilus* (*Bst*) which has strand displacement activity leading to DNA autocycling at a constant temperature of about 60-65^{o}C (does not require cyclical temperature change like NASBA) (Kumar *et al*., 2017). Unlike other nucleic acid- based techniques, it is a field-friendly technique that can be used at

point-of-care to detect low density malaria infections in malaria endemic settings (Morris *et al*., 2015; Tegegne *et al*., 2017). It displayed greater sensitivity and specificity than microscopy and RDTs. Ultrasensitive LAMP can able to detect parasites as low as 0.05-0.1 parasites/µl (Mohon *et al*., 2019).

Currently, in addition to the detection of *Plasmodium* parasites, LAMP is serving as a technique to identify molecular markers associated with drug resistance in *P. falciparum*. Different LAMP assays detected *P. falciparum* isolates carrying *Pfcrt* K76T (Chahar *et al*., 2017), C580Y (Imai *et al*., 2018; Khammanee *et al*., 2021) and N51I (Yongkiettrakul *et al*., 2020) mutations associated with CQ, ART and pyrimethamine resistance, respectively.

Although LAMP has advantages, it has also the following disadvantages (Sahoo *et al*., 2016): (i) it is useful primarily as diagnostic or detection technique but not serve for cloning purposes because the final product is a large DNA chain, (ii) problem in designing proper primers, (iii) primer dimer resulting from larger number of primers per target DNA and (iv) the product of LAMP displayed a characteristic "ladder" or banding pattern on a gel, rather than a single band as with PCR, thus identification of a target by size of the band on a gel is not possible with LAMP.

2.6. Malaria Control and Elimination Strategies

Malaria control aims at reducing the burden of the disease to a locally manageable level where it is no longer public health problem. Malaria elimination describes the process in which local malaria transmission is interrupted and the incidence of infection in defined geographic area is reduced sufficiently to prevent re-establishment of transmission (WHO, 2008a). Effective vector control and case management (prompt and effective treatment) strategies are the most powerful tools to control and eliminate malaria transmission (Benelli & Beire, 2017; WHO, 2022). These were reviewed in detail below.

2.6.1. Vector Control

Vector control is an essential component of malaria prevention and elimination in all epidemiological settings. Mosquito vector control strategies mainly relied on chemicals, biological, plant extracts and environmental management) (Raghavendra & Barik, 2011; Benelli & Beire, 2017).

Long-lasting insecticide treated nets (LLINs) are one of the most widely used and effective malaria vector control interventions (Bhatt *et al*., 2015; WHO, 2016a). They are the most important interventions across Africa. Between 2000-2015 sixty-eight percent of 663 million malaria cases were averted due to LLINs (Bhatt *et al*., 2015; Roux *et al*., 2021). Wide coverage of ITNs has put a remarkable selection pressure on *Anopheles* mosquitoes and resulted in the emergence and spread of resistance. However, resistance of pyrethroides is a recent phenomenon and it is currently the only classes of insecticides used to make LLINs (Awolola *et al*., 2018; MOH, 2020).

IRS is the application of long-acting chemical insecticides on the interior walls and roof to kill adult mosquitoes landing on the surfaces (Raghavendra & Barik, 2011; Gari & Lindtjørn, 2018). It is one of the core malaria intervention tools in addition to ITNs. It is estimated that 10% of 663 million of clinical cases were averted between 2000 and 2015 due to IRS (Bhatt *et al*., 2015).

Malaria vector control in SSA heavily depends on these indoor vector control interventions (LLINS and IRS). Significant gains by these interventions brought many malaria endemic countries to malaria pre-eliminations phase and enabled WHO and Roll Back Malaria to set plan of reducing global burden of malaria by 90% in 2030 (WHO, 2016b). Indoor insecticides are mostly effective for endophagic (indoor feeding) and endophilic (indoor resting) malaria vector species (Bhatt *et al*., 2015). However, the control of malaria is threatened by insecticide and behavioral resistant vectors and these may play significant roles in the existence residual malaria transmission (Asale *et al*., 2017; Gari & Lindtjørn, 2018).

Malaria vectors are rapidly developing resistance to all classes of insecticides (WHO, 2016a; Gari & Lindtjørn, 2018). The development of behavioural resitance also resulted in increased outdoor biting and resting proportions of *Anopheles* mosquitoes. A shift in biting time (from late to early evening hours) before people indoors and protected by bednets was reported from different African countries for certain vectors such as *An. arabiensis* and *An. funestus* and poses challenge to control malaria by only indoor interventions (Asale *et al*., 2017; Gari & Lindtjørn, 2018). This shift from endophilic to exophilic adapted species may be due to the application of extensive indoor vector controls (Benelli & Beier, 2017). Certain *Anopheles* mosquitoes such as *An. arabiensis* which in addition to human host take blood meals from

animals (zoophilic) and rest outdoors (exophilic) (Asale *et al*., 2017), can not be controlled only by indoor interventions.

As malaria continues to decline, asymptomatic infections persist without detection and treatment and become the source of mosquito infections in the community (Ouedraogo *et al*., 2009). Thus, for many malaria risk population, elilmination of residual malaria transmission is unachievable with only wide coverage of core interventions. Therefore, these challenges call for supplementory (outdoor-based) intervensions such as larvicides with temophose (abate) (Setyaningrum *et al*., 2021), *Bacillus thuringiensis israelensis* (Mutero *et al*., 2020), plant extracts (Tarekegn *et al*., 2021), zooprophylexis and environmental methods (Asale *et al*., 2017; Gari & Lindtjørn, 2018; Sougoufara *et al*., 2020). Futhermore, promising potential new tools for mosquito control are being developed, the most noticeable ones are "eartubes", ivermectin and attractive toxic sugar baits (ATSB). These methods are highly effective, target specific and with very less environmental contamination effects (Benelli & Beier, 2017; Sougoufara *et al*., 2020). In Mali, a single outdoor application of ATSB co-formulated with boric acid resulted in a 90% decrease of *An. gambiae s.l.* densities. Similarly, application of endectocide ivermectin over 48h reduced semi-field cage populations of *An. arabiensis* densities by 95% (Sougoufara *et al*., 2020).

2.6.2. Case Management and Anti-malarial Treatment

Malaria case management is a vital component of malaria control and elimination strategies (WHO, 2022). It consists of early diagnosis and prompt effective treatment to minimize morbidity and mortality, reduce transmission and unnecessarily use of anti-malarial drugs and thus reduce the emergence and spread of anti-malarial drug resistance (Ukpe *et al*., 2013; WHO, 2022). Early diagnosis and prompt effective treatment within 24-48h commencement of malaria symptoms can rapidly reduce the progression of uncomplicated malaria to severe malaria and death (WHO, 2015). A delay in treatment allows the patient to remain infectious even after being treated because gametocytes may persist for several weeks after the clearance of asexual stages (Bousema & Drakeley, 2011). At health facilities all patients suspected of malaria should be confirmed by microscopy examination or RDT blood sample test before the commencement of treatment (Basu & Sahi, 2017; WHO, 2021a).

ACTs are the recommended first-line drugs for the treatment of uncomplicated falciparum malaria in all endemic areas (WHO, 2022). Currently, WHO recommends six ACTs: artesunate-mefloquine (AS-MQ), artemether-lumefantrine (AL), artesunate-amodiaquine (AS-AQ), dihydroartemisinin-pepraquine (DHA-PPQ), artesunate-pyronaridine (AS-PA) and artesunate + sulfadoxine-pyrimethamine (AS-SP) for the treatment of uncomplicated falciparum malaria (WHO, 2022). ACTs have played a significant role in the decline of malaria related cases. As part of a significant reduction in malaria burden between 2000 and 2015, of the 663 million clinical cases of malaria averted 22% was contributed by ACTs (Bhatt *et al*., 2015).

In low transmission areas, ACT is recommended with a single gametocytocidal dose of PQ to patients (except pregnant women, infants aged < 6 months and women breast-feeding infants < 6 months) with uncomplicated falciparum malaria to reduce transmission without the need to test for Gulucose-6-phosphate dehydrogenase (G6PD) (White *et al*., 2013; WHO, 2022).

CQ or ACTs is recommended for uncomplicated malaria caused by other *Plasmodium* species: *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. For radical cure of *P. vivax* and *P. ovale* children and adults (except infants < 6 months, pregnant and breast feeding women and people with G6PD enzyme deficiency) of all transmission settings should be treated with PQ for 14 days (WHO, 2022).

2.6.2.1. Anti-malarial Drugs and Their Mechanisms of Action

Together with other interventions, anti-malarial drugs have been used to treat and prevent malaria for centuries. In this section the most commonly used anti-malarial drugs and their modes of action have been addressed. Based on their pharmacological activities, these anti-malarial drugs are categorized as: blood schizonticides, tissue schizonticides, gametocytocides and sporozontocides (Figure 2).

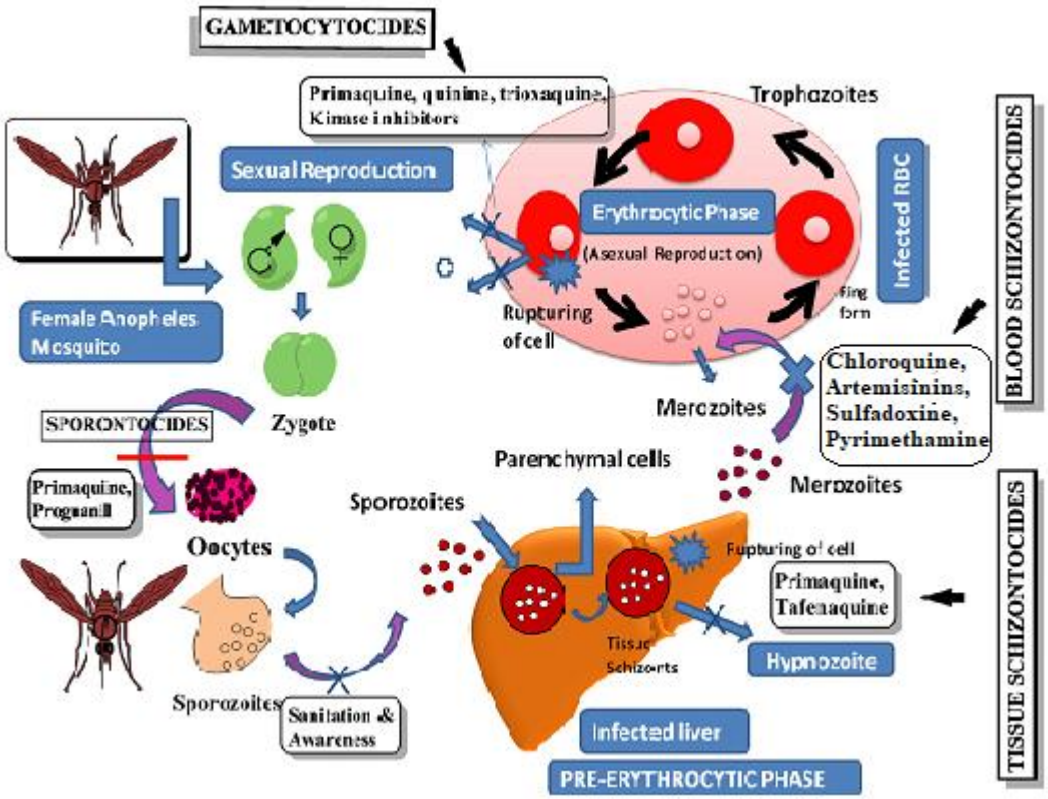


Figure 2. Anti-malarial drugs and their targets of action in the life cycle of *Plasmodium* parasites. Adapted from Kumar *et al*. (2018).

According to their chemical structure and modes of action, currently available drugs have been categorized into three main groups: quinolines, anti-folates and ART and its derivates.

Quinolines

There are two sub-classes of quinolines: aminoquinolines and aryl-aminoalcohols.

Aminoquinolines

This group includes 4-amonoquinolines (CQ, AQ, PA and PPQ) and 8-aminoquinolines (PQ, tefnoquine). They are weak bases that are deprotonated and hydrophilic at neutral P^H.

Chloroquine (CQ)

CQ was the most widely used anti-malarial drug due to low cost, safety and efficacy. As it is protonated, weak base drug, its accumulation in digestive vacuole (DV) causes the rise in P^H. It prevents polymerization and detoxification of hematin and thereby interferes with

breakdown of host erythrocytic haemoglobin and prevents *Plasmodium* growth (Zhou *et al*., 2020; Daskum *et al*., 2021). Its unwise use caused the spread of CQ resistant *P. falciparum*.

Amodiaquine (AQ)

AQ is structurally similar to CQ. Functionally also in similar way to CQ, it is hypothesized to form complex with haem and prevents haemazoin (malaria biomarker) crystal formation (Rout & Mahapatra, 2019; Tse *et al*., 2019). Currently, it is mainly used in combination with AS (AS-AQ) to treat uncomplicated *P. falciparum* malaria (WHO, 2021b).

Premaquine (PQ)

PQ is an 8-aminoquinoline pro-drug recommended for the radical cure (preventing relapse) of *P. vivax* and *P. ovale* (Daskum *et al*., 2021; WHO, 2021b). It is also the only drug of choice for the treatment of *P. falciparum* gametocytes and hence prevents/reduces onward transmission to mosquitoes (Mwaiswelo *et al*., 2022). Although its mode of action is not clearly elucidated, PQ treatment interferes with metabolic process of gametocyte mitochondria (Bousema & Drakeley, 2011). However, recently it was investigated that hydroxylated-PQ metabolites were able to kill mature gametocytes through increased oxidation and excessive generation and accumulation of H_2O_2 (Camarda *et al*., 2019). H_2O_2 exerts its killing effect of the parasite through oxidation proteins of sulphydryl groups and damaging of iron and iron-sulphide containing protein or through generation of superoxide and hydroxyl radicals.

Tafenoquine

Tafenoquine is a synthetic analogue of PQ which is long acting (eliminate slowly) antimalarial drug targeting liver stage of *Plasmodium* parasites. It was designed to be more stable than PQ and recently approved by the United States Food and Drug Admnistration for use as single dose for the radical treatment of *P. vivax* (Tse *et al*., 2019; Daskum *et al*., 2021). Although its mode of action is not elucidated, just like PQ it is pro-drug which is metabolized to active tafenoquine.

Aryl-amino alcohols

Aryl-amino alcohols are weak bases that are lipid soluble at neutral P^H. This group includes anti-malarial drugs such as qunine, MQ, HF and lumefantrine (LF).

Quinine (QN)

Quinine (QN) was the first quinoline drug used to treat malaria. It was first isolated from the bark of Cinchona tree (Daskum *et al*., 2021). Although its mode of action was not well elucidated, it was proposed to be similar to CQ where in the DV of the parasite it inhibits formation of hemazoine (non-toxic hematin). Due to its side effects and widespread quinine resistant *Plasmodium* parasites it is recommended only for the treatment of severe malaria (Tse *et al*., 2019; Daskum *et al*., 2021).

Other amino-alcohols are also blood schizonticides that target asexual stage of the parasites (Rout & Mahapatra, 2019). They disrupt haemoglobin digestion and inhibit haemazoin formation. Currently, MQ and LF in combination with AS and artemether, respectively are used for the treatment of uncomplicated falciparum malaria.

Anti-folates

Folate metabolism is a series of enzymatic activities involved in the synthesis of folate and its derivatives. These products (cofactors) are required for the synthesis of DNA, synthesis of amino acids (glycine and metheonine) and for metabolism of histidine, glutamic acid and serine by *Plasmodium* parasites (Daskum *et al*., 2021). Dihydrofolate reductase (DHFR) and dihydropetroate synthase (DHPS) are enzymes involved in this essential metabolic pathway of *Plasmodia*. Anti-folate drugs pyrimethamine and proguanil inhibit the activity of DHFR while sulfadoxine and dapsone drugs target DHPS enzymes. Combination therapy of sulfadoxine and pyrimethamine (SP) is more effective in inhibiting these enzymes than either of the drugs used as monotherapy (Kumar *et al*., 2018; Daskum *et al*., 2021). Point mutations in *dhps* and *dhfr* genes encoding these enzymes led to the spread of SP resistant *Plasmodium* parasites across the world (Saifi *et al*., 2013). Currently, SP is used as intermittent preventive treatment for pregnant women and infants and in combination with AS (AS-SP) for the treatment of uncomplicated falciparum malaria (WHO, 2021b).

Artemisinin and Its Derivatives

ART is an anti-malarial drug extracted from Chinese medicinal plant (herb) *Artemisia annua* (Chinese wormwood). It is a sesquiterpene trioxane lactone containing an endoperoxide bridge that is essential for its anti-malarial activity (Petersen *et al*., 2011; Rout & Mahapatra, 2019; Daskum *et al*., 2021). This group of anti-malarials includes ART, AS, artemether, arteether

and dihydroartemisinin. In systemic circulation except dihydro-artemisinin, ART and its derivatives are all pro-drugs and hydrolyzed into dihydro-artemisinin, a biologically active metabolite (Tse *et al*., 2019).

ARTs have broad stage specificity against nearly all asexual stages and sexual gametocytes and thereby reduce gametocytes transmission from humans to mosquitoes (O'Neill *et al*., 2010; Belete, 2020). Modes of action of ARTs are not well understood. However, the following mechanisms were hypothesized: (i) ART activation is thought to involve iron-catalyzed endoperoxide bridge with parasite digested haemoglobin. The reaction releases carbon-centered free radicals (toxic products) which alkylate parasite proteins and other biomolecules and finally cause death of the parasites (Petersen *et al*., 2011; Rout & Mahapatra, 2019). (ii) Others argued that ART is a strong inhibitor of *P. falciparum* phosphatidylinositol-3-kinase (*Pf*PI3K) (Mbengue *et al*., 2015). In the early stages of *P. falciparum* (ring stages) *Pf*PI3K forms complex with Kelch13 protein and produces polyubiquitination of PI3K which subsequently results in degradation of parasites' proteins. This lysis of protein results in the decrease level of phosphatidylinositol-3-Phospahte (lipid product) which is responsible for internal transport of proteins (Mbengue *et al*., 2015; Rout & Mahapatra, 2019). (iii) Still there is evidence that ART targets *P. falciparum* Ca^{2+} transporting adenosine triphosphatase-6 (*Pf*ATPase6). Upon ART binding to *Pf*ATPase6, Ca^{2+} binding site on the enzyme (ATPase6) is lost. Loss of Ca^{2+} pump results in the death of the parasite (Shandilya *et al*., 2013; Tse *et al*., 2019).

Their short half-lives and fast spread of ART resistant *Plasmodium* parasites results in the formulation of ACTs (Petersen *et al*., 2011; Rout & Mahapatra, 2019). ACT is composed of short acting ARTs (ART and its derivatives) and long acting partner drugs. ARTs clear parasitaemia rapidly (within three days) while partner drugs eliminate the remaining parasitaemia by their different modes of action (Petersen *et al*., 2011; Rout & Mahapatra, 2019). Thus partner drugs in ACTs reduce ART resistance by minimizing the time parasitaemia exposed to ART.

2.6.3. Malaria Vaccine

Malaria control becomes more effective if malaria control strategies are combined with malaria vaccines. Moreover, malaria vaccines are appropriate and cost effective strategies to

eliminate and ultimately help to eradicate malaria. Rationale of developing malaria vaccines is the observation that people living in malaria endemic areas develop clinical protective immunity despite the morphological and antigenic variations the parasites display throughout their life cycle (Greenwood, 2005).

Development of malaria vaccines comprises three types of vaccines targeting various stages in the lifecycle of malaria parasites (Riche & Soul, 2002): (i) pre-erythrocytic vaccines targeting sporozoite and liver stages, (ii) blood-stage malaria vaccines targeting asexual stages, and (iii) transmission blocking vaccines targeting the sexual and mosquito mid-gut stages (gametes, zygotes, ookinets and oocysts). Most of the candidate vaccines are at pre-clinical or in clinical trials (Duffy & Gorres, 2020). However, two malaria vaccine candidates: RTS, S/AS01 (marketed under the trade name Mosquirix™) and R21/Matrx-M (R21/MM) have shown better protective effects in young children in late clinical trials. Both are pre-erythrocytic vaccines targeting the circumsporozoite protein of the sporozoites (Laurens, 2020; Datoo *et al*., 2021).

RTS, S/AS01 (Mosquirix™) is the first vaccine and protein-based recombinant malaria vaccine targeting *P. falciparum* that has shown partial protection against falciparum malaria in young children. At phase III clinical trial in 11 sites of seven SSA countries the vaccine has shown efficacy of 56% and 36% against clinical malaria in children aged of 5-17 months after follow-up of one and two years, respectively (Laurens, 2020). Though the vaccine was not pre-qualified by WHO (does not fulfill the least vaccine efficacy of 75% set by WHO) it (phase IV) was introduced for the first time into three SSA countries: Ghana, Kenya and Malawi since 2019 as part of a large-scale pilot programme coordinated by WHO (Hogan *et al*., 2020). Based on the results of the pilot programme, since October 2021, WHO recommended this vaccine as the first licensed malaria vaccine for children at risk in SSA and in other regions with moderate to high transmission of falciparum malaria (WHO, 2021b).

R21/Matrix-M vaccine candidate is the first to pass the WHO's goal of achieving a vaccine with 75% efficacy against malaria by 2030. A randomized control trial of R21/MM vaccine candidate (phase IIb) which was administered to 450 children of aged 5-17 in Burkina Faso showed vaccine efficacy of 77% and 75% (compared with control) in high and low doses of MM adjuvants, respectively in the follow-up period of 12 months (Datoo *et al*., 2021). In

2020, in those children of Burkina Faso that vaccinated with the same vaccine at high-dose adjuvant (5μg R21 adjuvanted with 50μg Matrix-M) 80 % vaccine efficacy was attained (Datoo *et al*., 2022). Furthermore, agreement was reached between vaccine developers and funders to produce large quantities of the vaccine at the late of 2020s (Rosenthal, 2022).

2.7. Surveillance Methods of Anti-malarial Resistance

Anti-malarial resistance is defined as the ability of *Plasmodium* parasite strains to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to or higher than the recommended ones, but within the tolerance of the human subject (WHO, 2010b). Nowadays, monitoring anti-malarial drug resistance depends on three different and complementary approaches: *in vivo* efficacy (clinical), *in vitro*/*ex vivo* (phenotypical) and molecular markers. Each of these methods has their own advantages and disadvantages (Abdul-Ghani *et al*., 2014; Nsanzabana *et al*., 2018).

2.7.1. *In vivo* Efficacy Studies

In vivo assays are considered as gold standard for measuring the efficacy of anti-malarial drugs and their outcomes serve as basis for malaria control program in order to take treatment policy decisions. The measurement involves prescribing the required dose of anti-malarial drugs in mono or combination regimen to patients infected with uncomplicated *Plasmodium* parasites. On regular basis, WHO update methods to standardize measurements of anti-malarial efficacy (WHO, 2009). Based on the drug's half-life, patients who received treatments are followed by parasitology and clinical assessments for a fixed number of days (28 to 63 days), after which the treatment considered as successful or not (Abdul-Ghani *et al*., 2014; Nsanzabana *et al*., 2018). Therapeutic responses are classified as adequate parasitological and clinical responses, early treatment failure or late treatment failure (WHO, 2009). Conducting *in vivo* efficacy study is challenging particularly in resource limited malaria endemic countries because it requires budget, infrastructures, man power that followed the patients on regular basis and testing them both parasitologically and clinically (Abdul-Ghani *et al*., 2014; Nsanzabana *et al*., 2018). Although the information gathered from i*n vivo* studies is what is exactly required to make evidence based malaria treatment polices, due to the above mentioned constraints in low income malaria endemic countries *in vivo* studies were conducted at only a few sites and at infrequent intervals (Plowe, 2003). On the other hand, the outcomes of therapeutic studies are

influenced by confounding factors such as host immunity, parasite factors and pharmacokinetics and the results of *in vivo* tests do not necessarily reflect the true level of pure anti-malarial drug resistance (WHO, 2009).

2.7.2. *In vitro*/*Ex vivo* Methods

These are the means of measuring *P. falciparum* susceptibility to anti-malaria drugs phenotypically using fresh collected parasite strains from patients (*ex vivo*) or culture-adapted parasite isolates (*in vitro*) (Abdul-Ghani *et al*., 2014; Nsanzabana *et al*., 2018). These are exclusively the assessment of intrinsic sensitivity of *P. falciparum* to varying concentrations of anti-malarial drugs to determine parasite growth inhibition. The assays measure 50% inhibitory growth (IC_{50}) or survival rate of parasites compared to unexposed control groups (Witkowski *et al*., 2013). The outcomes of these assays can support the results of *in vivo* efficacy studies (WHO, 2009). These assays omit host factors such as immunity, pharmacokinetics and genetics that can confound assay results by removing parasites from the host and placing them in a controlled experimental environment. Additionally, many samples (including those at experimental stages) can be tested on the same sample or several drugs can be studied simultaneously (WHO, 2009).

However, the usefulness of *in vitro*/*ex vivo* assays is limited by factors including time consuming, laborious, requiring skilled personnel, infrastructures and high parasite densities (Abdul-Ghani *et al*., 2014). Moreover, the process of freezing and thawing repeatedly may cause the probability of selecting subpopulation of the parasites that ultimately genotypically or phenotypically unrepresentative of the original population (Plowe, 2003). IC_{50} assays which have been recently standardized by Worldwide Anti-malarial Resistance Net-work are not suitable for ART derivatives because of their short half-life. Moreover, ART resistance has been proposed to be confined to the ring stage of the parasites and would not be predicted to influence susceptibility at trophozoite or schizont stage (Woodrow *et al*., 2013; Nsanzabana *et al*., 2018). Therefore, ART derivatives are tested by *in vitro* ring survival assay (Witkowski *et al*., 2013; Apinjoh *et al*., 2019).

2.7.3. Molecular Markers-based Surveillance

Unlike *in vivo* and *in vitro*/*ex vivo* methods, molecular markers enable to track the emergence and spread of anti-malarial drug resistance parasites (Abdul-Ghani *et al*., 2014). Molecular-

based surveillance could help as early warning signal for anti-malarial drug resistance before the apparent of clinical treatment failures. Both *in vivo* and *in vitro* methods are not suitable for large studies of ant-malarial resistance especially at national level of malaria control programs. Collection, transport and storage of samples for molecular analysis are so easier compared to *in vitro* method (Abdul-Ghani *et al*., 2014; Nsanzabana *et al*., 2018). Recent advancement in molecular techniques enables to process large samples and identify markers associated with drug resistance in short time. These techniques enabled to identify molecular mechanisms related with resistance to the most widely used ant-malarial drugs through detecting SNPs or copy number variations of the target genes (Costa *et al*., 2017; Nsanzabana *et al*., 2018). These tests are nucleic acid-based analyses of small amount of parasite DNA from whole blood or DBS (Apinjoh *et al*., 2019). However, molecular markers-based surveillance is not without limitations. The techniques require expensive equipment, reagents, skilled expertise and results may not always associated with *in vivo* studies (Abdul-Ghani *et al*., 2014; Nsanzabana *et al*., 2018).

2.8. Antimalarial Drug Resistance Genes

Among antimalarial resistance genes known so far, two key transporter genes *Pfcrt* and *Pfmdr1* which are relevant to this study were reviewed in this subsection.

2.8.1. *Plasmodium falciparum crt* Gene

Pfcrt is a 13 exon gene on chromosome 7 and encodes 48kDa protein which is made up of 424 amino acids and 10-α helical trans-membrane domains (TMDs) oriented inside the DV membrane while C- and N-termini are oriented towards the cytoplasm (Fidock *et al*., 2000) (Figure 3). TMDs mediate binding and translocation of substrates across the DV's membrane. This transporter mediates influx/efflux antimalarial drugs to and from DV of the parasite (Shafik *et al*., 2020). DV is acidic lysosome-like organelle that facilitates the digestion of hemoglobin and produce amino acids which are exported into the parasite cytoplasm to produce polypeptides. *Pf*CRT expression starts in the ring stages and reaches maximum expression during the trophozoite stages. Bioinformatics analyses predict PfCRT to be a member of drug/metabolite transporter superfamily of electrochemical potential driven transporters (Bray *et al*., 2005). It plays essential role for the survival of the malaria parasites. It transports small peptides from the DV to cytoplasm, for amino acids metabolism by the

parasite and thereby prevents osmotic stress of this organelle. *Xenopus* oocyst expression system showed that PfCRT exports host derived residues 4-11 in length from the parasite DV and the protein does not behave as a non-specific transporter of other metabolites and/or ions. Peptide transporting role of PfCRT was confirmed by PfCRT inhibitors such as verapamil where the transport of the peptides out of DV inhibited by the presence of verapamil (Shafik *et al*., 2020).

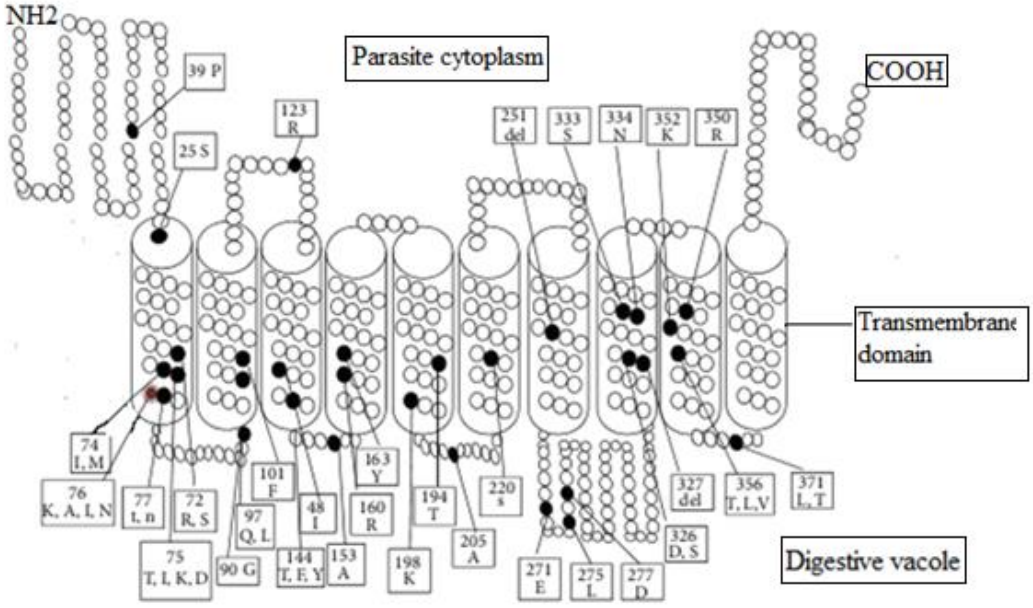


Figure 3. Detailed structure of PfCRT protein. It is made up of 422 amino acids distributed over 10 TMDs. Inside the structure there are 32 candidate codons for having point mutations that confer for changing PfCRT function. Seventy-five percent (24) of them occur at the site that faces the DV media. Adapted from Ibraheem *et al*. (2014).

2.8.1.1. Mechanism of *Pfcrt* as CQ Resistance

Pfcrt as much as important in the physiology of parasites it is also extensively involved in CQR (Shafik *et al*., 2020). CQR parasites accumulate less CQ in DV compared to CQS, because they efflux CQ via energy mediated process against concentration gradient (Wicht *et al*., 2020). Mutations in *Pfcrt* is the most probable mechanism of CQ reduction in DV (Wicht *et al*., 2020). However, the mechanism by which mutations in *Pfcrt* reduces accumulation of

CQ in DV is not yet concluded. Study conducted by Lehane & Kirk (2008) indicated that in the presence of CQ there is an increased leak of H^+ from the DV of CQR parasites compared to CQS ones. By using transfectant *Plasmodium* parasite strains that differ only by their *Pfcrt* allele, they showed CQR conferring *Pfcrt* mutations were responsible for CQ-associated H^+ leak, which means that *Pfcrt* mutations mediate the efflux of CQ in association with H^+ from the DV of the parasite. "Charged drug leak" model is an other mechanism proposed by which protonated CQ (CQ^{2+}) interact with PfCRT and diffuses passively out of DV down its concentration gradient (Bray *et al.*, 2006; Daskum *et al*., 2021). Others have argued that CQ actively transported out of DV by the expense of energy (Sanchez *et al*., 2007).

2.8.1.2. Polymorphisms in *Pfcrt* Gene

In vivo and *in vitro* studies have indicated that polymorphism in *Pfcrt* gene with substitutions at codon positions 72 to 76, 97, 152, 163, 220, 271, 326, 356 and 371 were associated with CQR (Djimde *et al*., 2001; Ibrahim *et al*., 2009; Ecker *et al*., 2012). However, todate not less than 32 point mutations have been identified in *Pfcrt* gene (Figure 3) that can alter both the phenotype and physiochemical of certain *P. falciparum* strains that confer them resistance to CQ (Ibraheem *et al*., 2014; Daskum *et al*., 2021). Of these polymorphisms, non-synonymous substitution of positively charged lysine with threonine at codon 76 (K76T) is a primary marker for CQR in field isolates (Fidock *et al*., 2000; Ecker *et al*., 2012). In laboratory adapted *P. falciparum* isolates treated with CQ a substitution of lysine with asparagine or isoleucine (76N/I) was detected in CQ resistant strains (Fidock *et al*., 2000). K76T a predominant mutation that occurred in CQ resistant *P. falciparum* parasites in Asia, South America and Africa (Fidock *et al*., 2000; Ecker *et al*., 2012). Field studies indicated that CQ treatment failure is associated with K76T *Pfcrt* mutation (Ecker *et al*., 2012).

Majority of mutations that have impact in physiochemical and phenotype on CQR parasites are the ones in *Pfcrt* within the codons between 72-76 (exon 2) (Ibraheem *et al*., 2014). These resulted evolutionary into three main haplotypes: CVMNK, CVIET and SVMNK. CVMNK, a CQ-sensitive haplotype, is found in CQ susceptible *P. falciparum* strains. CVIET and SVMNK haplotypes were evolved from CVMNK and displayed reduced susceptiblity to CQ. The former is found mostly in Africa and the later is mainly distributed in Southeast Asia and South America (Ibraheem *et al*., 2014; Ikegbunam *et al*., 2019; Adam *et al*., 2021; Roux *et al*.,

2021).

2.8.1.3. Dynamics of *Pfcrt* Gene Polymorpysim Post-ACTs Implementations

Due to the development and widespread of CQ and SP resistant *P. falciparum*, WHO recommended ACTs for the treatment of uncomplicated falciparum malaria. Malaria endemic countries adopted ACTs as first-line therapy since 2000. Before the official discontinuation of CQ several countries reported high frequencies and in some of them fixation of *Pfcrt* 76T (mutant-type) alleles (reviewed in Ocan *et al*., 2019).

After implementation of ACTs and cessation of CQ, reduction in the frequency of *Pfcrt* 76T alleles and the rise of wild-type K76 alleles are being reported from malaria endemic countries (Njiro *et al*., 2022). High frequencies of K76 alleles were reported from Tanzania (85%-93%) (Mohammed *et al*., 2013), Haiti (100%) (Elbadry *et al*., 2013) Malawi (99.9%) (Frosch *et al*., 2014), Ethiopia (84%) (Mekonnen *et al*., 2014), Grande Comores Island (81.5%), (Huang *et al*., 2016) Cote d'Ivoire (83.4%) (Konaté *et al*., 2018), Uganda (71% and 100%) (Balikagala *et al*., 2020), Zambia (97.3%) (Mulenga *et al*., 2021), Gabon (62.29% and 88.5%) (Tuedom *et al*., 2021) and Ghana (77.17%) (Asare *et al*., 2021). However, in other countries even though there is a gradual rise in K76 wild-type allele frequencies; the prevalence of 76T alleles is high and in some countries this allele is nearly fixed. High frequencies of 76T alleles were reported from Yemen (81.5%) (Al-Mekhlafi *et al*., 2011), India (72.13%), (Goswami *et al*., 2014), Ethiopia (100%) (Golassa *et al*., 2015), Eritrea (84.5%) (Menegon *et al*., 2016), Nigeria (94.56%) (Ikegbunam *et al*., 2019) and Angola (71.4%) (Ebel *et al*., 2021).

These differences in the rates of K76 rises and decline in 76T alleles in countries might be associated with when the countries adopted ACTs and terminated CQ treatment. For example, Malawi was the first African country to change its CQ treatment policy by 1993 (Kublin *et al*., 2003) while Ethiopia changed CQ policy five years later in 1998 (Golassa *et al*., 2015). In countries where withdrawal of CQ was strictly followed, it has been resulted in significant decreases in prevalence of CQ resistant parasites due to decrease in CQ drug pressure (Mohammed *et al*., 2013; Mulenga *et al*., 2021; Njiro *et al*., 2022). On the other hand, access to CQ might also contribute to the high frequency of this mutant allele. In some countries like Ethiopia (Golassa *et al*., 2015), Pakistan (Khan *et al*., 2020) and Bangladesh (Johora *et al*., 2021) CQ is used as first- line treatment for *P. vivax* which might expose *P. falciparum* to CQ

and as a result high prevalence of 76T is being reported from these countries. For example, high frequency of *Pfcrt* 76T mutation was reported from higher endemicity of *P. vivax* areas (Hailemeskel *et al*., 2019). Presumptive diagnosis, lack of diagnostic facilities and self-treatment of malaria by CQ and AQ may be also other contributing factors to the situation (Goswami *et al*., 2014; Tajebe *et al*., 2015; Balikagala *et al*., 2020; Khan *et al*., 2020; Adam *et al*., 2021).

ACTs based therapeutic efficacy studies were conducted to assess the prevalence of *Pfcrt* K76T polymorphisms in various malaria endemic countries. AL based therapeutic efficacy studies conducted by Sisowath *et al*. (2009) and Maiga *et al*. (2021) in Tanzania and Mali, respectively showed that parasites populations that carry K76 (CQ-sensitive) were selected more than those with 76T (CQ-resistant) allele in recrudescence and /or re-infections. On the other hand, AL and ASAQ exert opposing selective effects in *Pfcrt* gene. Therapeutic efficacy studies using AL and ASAQ conducted by Venkatesan *et al*. (2014) and Beavogui *et al*. (2021) in Mozambique and Republic of Guinea, respectively indicated that parasite populations exposed to AL were selected towards CQ sensitive (K76 allele) while ASAQ selected more parasites with 76T mutant allele in recrudescence and/or re-infections. Another AL and ASAQ therapeutic efficacy study conducted in Angola showed that most (92%) of ASAQ failure samples from recurrent parasitaemia carried the 76T *Pfcrt* allele. However, only 16% of AL failure samples had this allele (Dimbu *et al*., 2021). This preferential selection of 76T mutant allele by ASAQ may be because of the AQ partner drug which is structurally and chemically similar to CQ (Tinto *et al*., 2008). Therapeutic efficacy of AL is high in Ethiopia and above WHO's threshold (>90%) (Nega *et al*., 2016; Abamecha *et al*., 2020; Tadele *et al*., 2022), however, no *in vivo* study was done in association with *Pfcrt* K76T polymorphisms.

As in the case of *Pfcrt* 76 alleles, due to a reduction in CQ pressure, in some countries there is a reduction in the frequency of CQ-resistant haplotypes (CVIET and SVMNT) and a reversion of wild-type CVMNK haplotypes in high rates in *P. falciparum* isolates; recent sequencing of *P. falciparum* isolates from Africa revealed that 70% of them carried CVMNK haplotype (Veiga *et al*., 2016). For example, in Malawi, CVMNK haplotypes were persistently detected in the majority of clinical samples (Frosch *et al*., 2014). At Southeast Tanzania study conducted by Bwire *et al*. (2020) showed that 99.8% of the *P. falciparum* isolates were found harbouring CVMNK haplotype. Very few CVIET haplotypes carrying *P. falciparum* isolates

were found at Northern Uganda (Balikagala *et al*., 2020). Similarly, high frequencies of CVMNK CQ sensitive haplotypes were observed in Ethiopia (Mekonnen *et al*., 2014). However, predominance and in some cases fixation of CVIET haplotyes were reported in clinical samples from countries such as Ethiopia (Golassa *et al*., 2014), Yeman (Atroosh *et al*., 2016), Nigeria (Ikegbunam *et al*., 2019; Adam *et al*., 2021) and Southwestern Uganda (Kassaza *et al*., 2021). In Southeast India a dominance of SVMNT haplotypes were detected in clinical samples (Antony *et al*., 2016).

2.8.2. *Plasmodium falciparum mdr1* Gene

The *Pfmdr1* is a structural homologue of the mammalian multidrug resistance gene encoding P-glycoprotein 1 multidrug resistance transporter and it is expressed into *Pf*MDR1 protein located on the *P. falciparum* DV (Foote *et al*., 1990). *Pf*MDR1 is a protein with 1419 amino acids in length (for the 3D7 isoform) (Veiga *et al*., 2016).

*Pf*MDR1 protein is a trans-membrane protein (Figure 4), with two domains each consisting of 6 helical TMDs and a nucleotide binding fold region that act as a site for adenosine triphosphate (ATP) binding, and belongs to the ATP-binding cassette super family. The binding domains (NBD1 and NBD2) are located towards the cytoplasm where they interact with anti-malarial drugs and ATP (Ibraheem *et al*., 2014; Wicht *et al*., 2020). It acts as efflux pumps that help in substrate translocation including the antimalarial agents and have been linked to multidrug resistance in malaria (Veiga *et al*., 2016). This gene is expressed during blood stage of the parasite, reaching maximum peak by the end of the schizont phase just before the rupture of RBCs (Gil & Krishna, 2017).

Five globally prevalent amino acid mutations have been identified in *Pfmdr1* gene (Figure 4). The amino-terminal mutations (N86Y and Y184F) are more common to African and Asian parasites; whereas the carboxyl-terminal mutations (S1034C, N1042D and D1246Y) are found frequently in South American parasites (Ibraheem *et al*., 2014; Veiga *et al*., 2016). However, genome sequencing of *P. falciparum* parasites from Africa by the MalariaGEN consertum revealed that D1246Y is present in ~3% of 1,502 genomes (Veiga *et al*., 2016). These mutations altered the physiochemical of *Pf*MDR1 protein as the substitute amino acids are more polar compared to their substituent and thereby affects its potential to bind to and transport different molecules (Ibraheem *et al*., 2014). These SNPs are associated with

modulations of parasites tolerance or susceptibility to a number of anti-malarial drugs including QN, CQ, MQ, AQ and CQ (Sanchez *et al*., 2010).

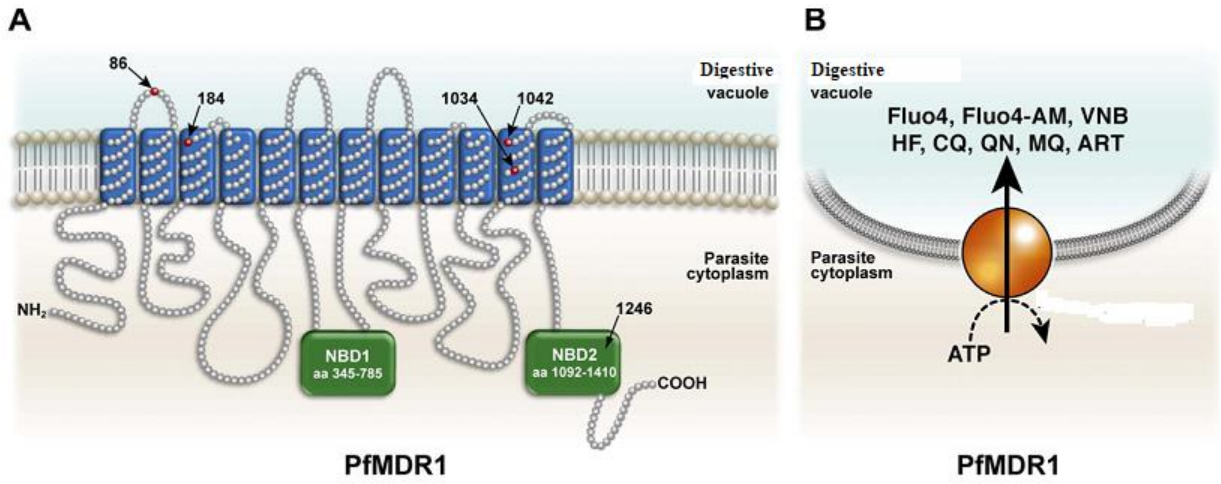


Figure 4. Structure (A) and function (B) of *Plasmodium falciparum* multi-drug resistance 1 (PfMDR1). (A) Arrows indicate the position of five polymorphic amino acids. NBD, nucleotide binding domain (B) proposed substrate for PfMDR1. PfMDR1 seems to pump compounds including fluorochromes (Fluo4, Fluo4-AM) into DV. CQ, chloroquine; AQ, amodiaquine; QN, quinine; MQ, mefloquine; HF, halofantrine; ART, artemisinin; ATP, adenosine triphosphate. Source: Sanchez *et al*. (2010).

2.8.2.1. *Pfmdr1* Polymorphisms in Anti-malarial Drug Resistance

2.8.2.1.1. Chloroquine Resistance *Pfmdr1* Mutations

The involvement of *Pfmdr1* mutations in CQR was evidenced for the first time by the study of Reed *et al*. (2000). Polymorphisms of *Pfmdr1* modulates the level of *in vitro* CQR in parasites that already harbor *Pfcrt* 76T mutation (Reed *et al*., 2000). The relevance of variations caused by *Pfmdr1* mutations in IC_{50} values within CQR cannot be generalized to the extent of parasite responses *in vivo* and the role of *Pfmdr1* in CQ treatment failure remains unclear.

Pfmdr1 N86Y and D1246Y mutations are cited as potential contributors for CQR (Laufer & Plowe, 2004; Okell *et al*., 2018). However, epidemiological study failed to link *Pfmdr1* N86Y expression level with spread of CQR in the area (Ibraheem *et al*., 2014). Similarly, N86Y mutation was not associated with CQ response by *in vitro* experiment (Wurtz *et al*., 2014). On

the other hand, the replacement of N86Y mutation by N86 wild-type allele in *P. falciparum* lines resulted in increased susceptibility to CQ (Veiga *et al*., 2016). Conversely, N86Y mutation decreases parasite susceptibility to CQ (Mwai *et al*., 2009) and the effect is significant in parasites expressing CVIET PfCRT variant (Veiga *et al*., 2016). *In vitro* studies conducted by Shrivastava *et al*. (2014) and Lucchi *et al*. (2015) in India and Kenya, respectively indicated that *P. falciparum* parasites carrying both *Pfcrt* 76T and *Pfmdr1*-86Y mutations had significantly higher CQ IC_{50} values compared to the wild-type N86 allele. The two genes displayed strong linkage disequilibrium (Shrivastava *et al*., 2014). This argument was also supported by CQ and AQ efficacy studies conducted by Djimde *et al*. (2001) and Tinto *et al.* (2008) where N86Y mutation occurred along with K76T mutation in samples obtained from patients with post-treatment falciparum infections.

Transfections of *Pfmdr1* S1034C, N1042C and D1246Y mutations into CQS parasites had no effect on parasites sensitivity to CQ; suggesting that *Pfmdr1* polymorphisms are insufficient to confer CQR (Reed *et al*., 2000). *Pfmdr1* mutations may play a secondary role in CQ treatment outcomes but they are not sufficient to cause CQ treatment failures (Laufer & Plowe, 2004).

2.8. 2. 2. Patterns of *Pfmdr1* Polymorphisms Post-ACTs Implementation

Prior to the implementation of ACTs and cessation of CQ high prevalence of one or two or three of 86Y, 184Y and 1246Y alleles were reported from malaria endemic countries such as Uganda (Dokomajilar *et al*., 2006), Zanzibar (Sisowath *et al*., 2007), Sudan (Gadalla *et al*., 2013) and Pakistan (Khan *et al*., 2020), suggesting the presence of AQ or CQ quinoline drug pressure. 86Y/Y184/1246Y (YYY) and 86Y/1246Y (YY) haplotypes were also the commonest before the introduction of ACTs (Kublin *et al*., 2003).

After adoption of ACTs for the treatment of uncomplicated falciparum malaria, molecular marker-based surveillances showed that the prevalence of N86, 184F, D1246 alleles and the corresponding haplotype N86-184F-D1246 (NFD) are increasing due to AL selection (Adamu *et al*., 2020; Khan *et al*, 2020; Mulenga *et al*., 2021). In contrast, the prevalence of the YYY haplotype is on declining (Malmerg *et al*., 2013). Comparative impact of AL on *Pfmdr1* over 5 years in Uganda showed that the prevalences of *Pfmdr1* N86, 184F and D1246 increased over time (Conrad *et al*., 2014). N86/Y184/D1246 (NYD) is an other highly prevalent haplotype being selected with AL (Dimbu *et al*., 2021; van Loon *et al*., 2021). Since AL and AS-AQ

exerted opposite trends in selecting *Pfmdr1* alleles (Sondo *et al*., 2016), in countries where AS-AQ is used for the treatment for falciparum malaria the 86Y, Y184 and Y1246 alleles and YYY haplotype are being selected (Sisowath *et al*., 2007; Okell *et al*., 2018; Beavogui *et al*., 2021) because AQ as partner drug in this combination is structurally similar to CQ (Tinto *et al*., 2008). Similar trends were also observed for *in vitro* and *ex vivo* studies (Dahlström *et al*., 2014; Wurtz *et al*., 2014).

N86, 184F, D1246 alleles and NFD haplotypes which are increasing in prevalence after the implementation of AL might be due to their decresed susceptibility to the drug (Malmberg *et al*., 2013; Venkatesan *et al*., 2014; Veiga *et al*., 2016; Chidimatembue *et al*., 2021). *In vivo* susceptibility study conducted by Malmberg *et al*. (2013) in Tanzania indicated that re-infecting parasites with *Pfmdr1* NFD haplotype were able to tolerate LF blood concentration 15-fold higher than those with YYY haplotype (associated with decreased susceptibility to ASAQ (Venkatesan *et al*., 2014)). The largest difference was observed for parasites carrying N86 wild-type allele that withstand 12-fold higher LF concentration compared to those with 86Y allele. Similarly, allelic replacement study conducted by Veiga *et al*. (2016) revealed that substitution of N86Y mutation with N86 wild-type withstand 3-4 fold higher IC_{50} concentration of LF and MQ compared to 86Y residues. This finding is also in line with therapeutic efficacy study where parasites harbouring *Pfmdr1* N86 tolerate higher LF levels and have short-time to re-infection or recrudescence in patients with high LF concentrations in patients treated with AL (Otienoburu *et al*., 2016). Conversely, replacement of N86 with 86Y was able to increase parasite susceptibility to DHA-PPQ and monodesethyl-AQ (active metabolite of AQ).

Others *in vitro/Ex vivo* studies also revealed the selection of these alleles/haplotypes by AL. *Ex vivo* efficacy study conducted by Dahlström *et al*. (2014) in Benin, showed that significant difference of *Pfmdr* N86 wild-type allele was selected after AL treatment compared to the baseline treatment. Similarly, *in vitro* studies conducted by Mwai *et al*. (2009) and Wurtz *et al*. (2014) in Kenya revealed that N86 wild-type allele was associated with decreased susceptibility to LF and increased susceptibility to AQ. N86Y mutation showed increased susceptibility to LF and decreased susceptibility to AQ (Wurtz *et al*., 2014).

Directional selection of *Pfmdr1* alleles by ACTs can be evidenced also by therapeutic efficacy

studies. An increase of N86 wild-type allele in recrudescence and/or re-infections after AL therapeutic efficacy studies was reported from countries such as Liberia (Otienburu *et al*., 2016), Burkina Faso (Sondo *et al*., 2016) and Angola (Dimbu *et al*., 2021), Republic of Guinea (Beavogui *et al*., 2021) and Mali (Maiga *et al*., 2021). The selection of N86 may represent a marker of tolerance to LF (Veiga *et al*., 2016). Meta-analysis of 31 clinical trials from Africa, Asia/Oceania showed that patients infected with N86 parasites had a five-fold greater risk of parasite recrudescence following AL treatment compared with those infected with N86Y parasites (Venkatesan *et al*., 2014). In the recent low efficacy AL (below the WHO threshold) reported from Angola it was shown that N86 allele was found in 97% of treatment failures (Dimbu *et al*., 2021). In contrast to AL, AS-AQ based therapeutic studies have shown the selection of 86Y mutant allele in recrudescence and/or re-infections (Sondo *et al*., 2016; Beavogui *et al*., 2021).

With regards to *Pfmdr1* Y184F, post-ACTs implementations in several studies Y184 and 184F are selected respectively by AL and ASAQ in recrudescence and/or re-infections (Sisowath *et al*., 2007; Venkatesan *et al*., 2014; Otienburu *et al*., 2016; Ippolito *et al*., 2020). For example, therapeutic efficacy of AL conducted by Venkatesan *et al*. (2014) indicated that prevalence of *Pfmdr1* 184F increased by 2.1 fold in re-infections. However, its role in mediating drug sensitivity is unclear. Compared to N86Y mutation, Y184F mutation has weaker association with anti-malarial effectiveness (Veiga *et al*., 2016). Y184F did not show significance difference in *in vitro*/e*x vivo* susceptibility to anti-malarials tested (Duraisingh *et al*., 2000; Balikagala *et al*., 2020; Fukuda *et al*., 2021). Its weaker relationship with drug susceptibility indicates that by itself does not cause the failure of the drug treatment. Its drug susceptibility may depend on the presence of N86 residue; for example therapeutic efficacy study conducted by Ippolito *et al*. (2020) showed that 184F mutation reduced LF susceptibility most likely in the presence of N86 allele.

C-terminal *Pfmdr1* polymorphisms (S1034C, N1042D and D1246Y) also display reduced susceptibility to MQ, HF, LF and ART (Reed *et al*., 2000). These mutations are uncommon in Africa (Mekonnen *et al*., 2014; Veiga *et al*., 2016). However, in countries that Adopt AL there is rapid selection of wild-type D1246 mainly in East Africa countries where there is rapid decline in 1246Y mutation (Conrad *et al*., 2014; Okell *et al*., 2018). For example, in Rwanda prevalence of 1246Y mutation was decreased by 5-fold nearly over a decade (van Loon *et al*.,

2021). In countries where AS-AQ is implemented there is selection of this mutant allele (Beavogui *et al*., 2021). Therapeutic efficacy studies showed selection of D1246 and 1246Y alleles in recrudescence/re-infections respectively, by post AL and AS-AQ treatment (Sisowath *et al*., 2007; Venkatesan *et al*., 2014; Otienburu *et al*., 2016).

In addition to SNPs, *Pfmdr1* gene amplification (high copy number) is also associated with resistance to HF, MQ, ART and LF (Ibraheem *et al*., 2014; Okell *et al*., 2018). Meta-analysis of clinical trials showed that in addition to N86 wild allele, increased copy number was also a significant independent risk factor for recrudescence for patients treated with AL (Venkatesan *et al*., 2014). Parasites with increased copy number of the gene are rare in Africa (Venkatesan *et al*., 2014; Okell *et al*., 2018) but common in Southeast Asia where MQ was extensively deployed (Venkatesan *et al*., 2014). As LF is an aryl amino alcohol like MQ, there may be probability for the increased in copy number in parasites from Africa where AL is adopted as reported by some studies from Ethiopia (Tajebe *et al*., 2015; Lo *et al*., 2017).

In Ethiopia before the introduction of ACT particularly AL, there is no baseline data regarding the prevalence of *Pfmdr1* SNPs. However, few years post AL implementation studies showed low level of 1246Y allele and moderate to high levels of 86Y mutant allele (Schunk *et al*., 2006; Mula *et al*., 2011). There are some molecular marker based studies which show prevalence of these SNPs post-adoption of AL for uncomplicated falciparum malaria. Studies from various parts of Ethiopia showed that majority of *P. falciparum* isolates carried wild-type N86 allele (Golassa *et al*., 2015; Heuchert *et al*., 2015; Lo *et al*., 2017; Hailemeskel *et al*., 2019). High prevalence of 184F mutant allele was reported by Heuchert *et al*. (2015) and Lo *et al*. (2017). Conversely, high rate of Y184 sensitive wild-type allele was reported by Mekonnen *et al*. (2014). Regarding C-terminal *Pfmdr1* SNPs, predominance of wild types was reported by Mekonnen *et al*. (2014) and Heuchert *et al*. (2015). High prevalence of N86, Y184F, S1034, N1042 and D1246 alleles in Ethiopia indicate that these alleles are being selected under AL drug pressure.

Amplification of *Pfmdr1* gene is one mechanism of by which parasites reduced sensitivity to anti-malarials as discussed above. *Plasmodium falciparum* isolates with high copy number of *Pfmdr1* gene were reported from various parts of Ethiopia suggesting the impact of LF (partner drug of AL) drug pressure (Tajebe *et al*., 2015; Lo *et al*., 2017).

2.9. *Plasmodium falciparum* merozoite surface protein 1 (*PfMSP*1)

Effective invasion of human erythrocytes by *P. falciparum* is required for host invasion and the survival of the parasite. During the early stage of parasite, host invasion is mediated by merozoite surface proteins (MSPs) that interact with human erythrocytes. The most abundant MSP is glycosylphosphatidylinositol-an anchored called MSP1 (Dijkman *et al*., 2021). It is a 190kDa protein that is enzymatically cleaved by *Pf*subtilisin like protease into four fragments (p42, p38, p30, and p83) just before released from mature schizogonts. MSP1 also interacts with other merozoite surface proteins such as MSP3, MSP6, MSP7, and MSP Duffy binding Like 1 and 2 to bind to erythrocytes (Lin *et al*., 2016).

Merozoite surface protein-1 is the blood-stage vaccine candidate (Halder, 2009). The gene (*Pfmsp1*) that encodes this protein is comprised of 17 blocks in which some sequences are conserved (1, 3, 5, 12 and17), semi-conserved (7, 9, 11, 13 and 15) or variable (2, 4, 6, 8, 10, 14 and 16) (Bahrti *et al*., 2012). Among the seven variable blocks, block-2, sequence near the N-terminal of the *Pfmsp1*-gene, is the most polymorphic region. It is comprised of three allelic families: K1, MAD20 and RO33 (Bahrti *et al*., 2012; File *et al*., 2021). This block is under the strongest diversified selection within the natural population. Polymorphism in length and sequence diversity make this block target for the study of genetic diversity and multiplicity of infection in clinical *P. falciparum* isolates.

2.10. Transmission of *Plasmodium* Parasites from Human to Mosquitoes

Transmission of *Plasmodium* parasites starts with the presence of mature male and female gametocytes in the peripheral blood of infected individuals. During a blood meal, *Anopheles* mosquitoes must ingest at least one male and female mature gametocyte stages to become infected. The ingested gametocytes can be developed to ookinate and oocyst in the mosquito's mid-gut and finally to sprozoites in salivary glands that render mosquitoes infectious during the subsequent blood feeding (Musiime *et al*., 2019).

An estimate of onward transmission of *Plasmodium* reservoir to mosquito helps to understand epidemiology of malaria and to evaluate the efficacy of TRIs such as transmission blocking vaccines and gametocytocidal drugs (Miura *et al*., 2016; Stone *et al*., 2017; Challenger *et al*., 2021). The estimate of onward transmission depends on mosquito feeding assays where the mosquitoes are allowed to feed on blood from infected individuals (Bousema *et al*., 2012;

Miura *et al*., 2016). Malaria diagnostics including molecular techniques do not adequately predict the infectiousness of gametocyte infected individuals to mosquitoes (Bousema & Drakeley, 2011). Mosquito feeding assays are the only currently employed reliable tools to measure the infectiousness of gametocytes.

Mosquito feeding assays measure the transmission of gametocytes to mosquitoes by making mosquitoes to feed either directly on the blood infected individual or cultured gametocytes and then enable to assess infection rates in mosquitoes by oocyst and sporozoite detection (Bousema & Drakeley, 2011; Bousema *et al*., 2012). Currently, there are three mosquito feeding assays: direct SFA, DMFA and standard membrane feeding assay (SMFA) each of which has its own strengths and weaknesses (Bousema *et al*., 2013; Meibalan & Marti, 2017).

In SFAs mosquitoes are placed directly on the skin of malaria infected host and allowed to take blood (Bousema *et al*., 2012; Bousema *et al*., 2013). As it approximates natural means of mosquito feeding, it may have higher efficiency of feeding and yields higher proportion of infected mosquitoes compared to DMFA (Bousema *et al*., 2012). Compared to DMFA less number of mosquitoes is involved and gametocyte quantification is not as straightforward as in the case of DMFA, where DMFA allows gametocyte quantification from a portion of blood fed to mosquitoes, in the case of SFA gametocyte quantification is done from portion of blood on which mosquitoes are not fed. Furthermore, for ethical reason SFA studies do not involve young children (Bousema *et al*., 2012; Gaye *et al*., 2015).

The SMFA is a laboratory-based where *in-vitro* cultured gametocytes are mixed with compounds or test serum and fed through membrane feeders to laboratory reared *Anopheles* mosquitoes (Bousema *et al*., 2013). The assay measures the transmission blocking activity of antibodies by feeding cultured *P. falciparum* to *Anopheles* mosquitoes in the presence of test antibodies in serum or purified immunoglobulins and then measuring subsequent mosquito infectivity after one week (Miura *et al*., 2013; Bousema & Drakeley, 2017). Therefore, it is the gold standard technique for practical evaluation of antibodies in transmission blocking vaccines and has been commonly used in both preclinical and clinical studies (Miura *et al*., 2016). However, *P. vivax* cannot be cultured in the laboratory; therefore, parasites must be obtained directly from patients (Sattabongkot *et al*., 2015). Another limitation of SMFA is that it involves only few culture adapted strains of *P. falciparum* (Bousema *et al*., 2013).

DMFA involves feeding uninfected female *Anopheles* mosquitoes (wild or laboratory reared) on venous blood immediately after collected from malaria infected participants. The blood is offered to mosquitoes through water-jacketed glass feeders attached in series to circulatory water bath which is used to maintain optimum temperature for gametocytes. Mosquitoes are allowed to access blood through natural membranes such as Baudruche membrane or artificial membrane (Parafilm) (Bousema *et al*., 2013; Ouedraogo *et al*., 2013; Sattabongkot *et al*., 2015). It is a commonly employed assay in field settings to study onward transmission of malaria to mosquitoes. However, DMFAs are resource intensive, require an insectary and depend on huge logistics for blood sample collection, transportation and performing DMFA. The assay should be performed as soon as possible once the blood is collected from patients where the delay to perform DMFA can influence the outcome most probably due to premature gametocyte activation (Sattabongkot *et al*., 2015; Soumare *et al*., 2021).

Although DMFA is widely used, the condition under which it is performed is not yet standardized. Coulibaly *et al*. (2017) evaluated membrane type, starvation times, and mosquito age in *An. clouzzi* mosquitoes. Younger mosquitoes of 3 days ages are significantly associated with higher feeding, survival and infection rates as compared to 6 and 9 days aged mosquitoes. Longer starvation times were positively associated with infection rates, but negatively associated with feeding and survival. Baudruche membrane and Parafilm based feeding did not result in significant difference in feeding rate. However, another optimization of feeding rate of *An. farouti senso stricto* colony of mosquitoes conducted by Timinao *et al*. (2021) showed that Baudruche membrane based feeding yielded significantly higher feeding rate compared to mosquito fed through Parafilm membrane. This difference may be because of variation in physiological aggressiveness in feeding between the two species of mosquitoes used by two studies (Bousema *et al*., 2013). According to Vallejo *et al*. (2016) DMFA optimization study showed that best infectivity was attained in four day old mosquitoes fed at a density of 100 per cage. A protocol developed by Ouédraogo *et al*. (2013) showed a minimum starvation time of 5 hours before feeding. Generally, conditions which were optimized for particular species of mosquitoes may not exactly work for other groups of mosquitoes.

Mosquito infectivity studies were conducted in different malaria transmission intensities. Mosquito infectiousness is more prevalent in areas with high malaria transmission intensity

compared to low endemic regions of Burkina Faso (Gonçalves *et al*., 2017). From intense malaria transmission area of Burkina Faso, sub-microscopic individuals were responsible for 28.7% infectious individuals and 17.0% of mosquito infections (Ouédraogo *et al*., 2016). In an area of seasonal transmission of Burkina Faso, sub-microscopical *P. falciparum* gametocyte carriers were responsible for substantial (24.2%) malaria transmission in the study population (Ouédraogo *et al*., 2009). In low transmission areas of Cambodia, symptomatic patients of *P. falciparum* with microscope detectable gametocytes were more infectious to mosquitoes than those with microscope undetected gametocytes (Lin *et al*., 2016). In an area of hypoendemic malaria transmission in Senegal, similar level of infectivity was also observed (Gay *et al*., 2015). A recent symptomatic mosquito infectivity study conducted in Papua New Guinea showed that *P. vivax* gametocytemic infections were found more infectious to mosquitoes compared to those of *P. falciparum* (Timinao *et al*., 2021). This difference in infectivity may be because of difference in biological developments between gametocytes of the two *Plasmodia* species (Bousema & Drakeley, 2011; Nilsson *et al*., 2015).

Relative contribution of symptomatic and asymptomatic infections to infectious reservoirs was conducted in low-endemic setting of Ethiopia by (Tadesse *et al*., 2018). The study showed that *P. vivax* symptomatic infections were more infectious to mosquitoes compared to asymptomatic microscopic infections. For *P. falciparum,* more mosquito infections were from asymptomatic infections.

2.10.1. Factors Influencing Transmissions of *Plasmodium* Parasites to Mosquitoes

Transmission of *Plasmodium* parasites to mosquitoes can be influenced by factors including gametocyte density, presence of mature gametocyte, sex ratios, host and vector immune responses and interactions with other microorganisms in the gut of the mosquitoes (Bousema & Drakeley, 2011; Bousema *et al*., 2012; Bradley *et al*., 2018).

Infections with high gametocyte densities usually result in higher mosquito infections (Ouedraogo *et al*., 2009; Bousema *et al*., 2012; Abduselam *et al*., 2016; Bradley *et al*., 2018), however; this is not always the case where those infections with high gametocyte densities may not result in mosquito infections (Bousema & Drakeley, 2011; Abduselam *et al*., 2016). On the other hand submicroscopic malaria infections could also infect mosquitoes provide that both male and female mature gametocytes are present in the blood meal but at level undetected

by microscopy (Schneider *et al*., 2007; Bousema & Drakeley, 2011; Ouédraogo *et al*., 2016 Tadesse *et al*., 2018).

Mathematical models were developed by taking data of MFA on gametocyte carriers from different malaria endemic countries and tried to map the relationship between gametocyte density and mosquito infections and estimate reservoir of infection (Churcher *et al*., 2013; Bradley *et al*., 2018). The model developed by Churcher *et al*. (2013) which was based on the detection of female gametocyte density using *Pf*s25 marker showed that relationship between gametocyte density and mosquito infection was found to be non-linear. The other model was based on quantifying both male and female gametocytes using *Pf*s25 and *Pf*MGET markers, respectively (Bradley *et al*., 2018). The authors further suggested that quantifying both male and female gametocytes enable to correctly predict the relationship between gametocyte densities and success of mosquito infections than quantifying only female gametocytes. Although these findings need further investigations, they have important implications in gametocyte diagnostics.

Infectivity of gametocytes to *Anopheles* mosquitoes also depends on the presence of mature male and female gametocytes in the mosquito blood meal. In the case of *P. falciparum*, immature gametocyte stages (I-IV) sequester in deep tissues such as bone marrow; only stage V appears in peripheral circulation where they require additional 2-3 days to become fully mature and infectious to mosquitoes (Bousema & Drakeley, 2011; Nilsson *et al*., 2015). Incident falciparum infections are less infectious to mosquitoes compared to chronic asymptomatic infections (Barry *et al*., 2021) implying that patients with incident symptomatic falciparum infections require treatment before gametocytes become fully mature (Tadesse *et al*., 2018). In the case of *P. vivax* mature and infective gametocytes appear in peripheral circulation within 2-3 days after the appearance of asexual parasites (Bantuchai *et al*., 2022).

Another parameter that has an effect on gametocyte infectiousness to mosquito is gametocyte sex ratio (ratio of the number of male gametocytes to female gametocyte) (Robert *et al*., 2003; Mitri *et al*., 2009; Bradley *et al*., 2018). Gametocyte sex ratio is generally female-biased; however to maximize chance of fertilization male gametocytes produce up to eight microgametes to fertilize female gametes produced from single female gametocytes. Ratio of 3 or 4 female gametocytes to 1 male gametocyte is the usual occurrence in *Plasmodium*

infections which may fluctuate during course of infections or as a result of immune responses that selectively inhibit male and female gametocytes (Robert *et al*., 2003; Bousema & Drakeley, 2011). Male biased sex ratios showed higher transmission efficiency to mosquitoes. However, its transmission success depends on gametocyte densities. Models and MFA studies on infectivity of *P. falciparum* showed that infections with higher proportions of male gametocytes (male-biased sex ratios) gave higher transmission efficiency at low gametocyte densities but declined at higher gametocyte densities (Robert *et al*., 2003; Mitri *et al*., 2009; Bradley *et al*., 2018). This indicates rather than relying on total gametocyte density for success of infectivity, quantifying of both male and female gametocytes enables for better predictions of mosquito infectivity and to evaluate TRIs (Stone *et al*., 2017).

Success of gametocyte transmissibility and parasite development can be influenced by the presence of gametocyte specific antibodies present in the blood meal taken up by the mosquitoes (Bousema *et al*., 2011; Stone *et al*., 2018). Naturally occurring transmission reducing activity (TRA) against *P. falciparum* gametocyte has been associated with antibodies against *Pf*s48/45 and *Pf*s230 antigens (vaccine candidates) expressed internally and externally in gametocytes and gametes, respectively (Bousema *et al*., 2011; Stone *et al*., 2018). These antibodies prevent contact between male and female gametocytes (antibody for *Pf*s48/45) or reduce oocyst development by complement mediated lysis of gametes (antibody for *Pf*s230) (Bousema & Drakeley, 2011). DMFA and SMFA studies showed that individuals who exhibit high level TRA had higher density of antibodies for *Pf*s230 and *Pf*s48/45 antigens (pre-fertilization antigens) (Stone *et al*., 2018). The proportion of infected mosquitoes that became infected decreased with increasing in antibody density for *Pf*s230 and *Pf*s48/45 antigens in those individuals. In addition to *Pf*s230 and *Pf*s48/45, 43 novel proteins associated with high antibody mediated TRA were also investigated. These proteins may also elicit high level antibody responses in individuals sero-negative for *Pf*s230 and *Pf*s48/45 (Stone *et al*., 2018). Similarly, antibodies against *Pv*25 and *Pv*28 antigens significantly reduced oocyst development in *P. vivax* isolates may be by the mechanisms described for antibodies against *P. falciparum* (Bousema and Drakeley, 2011).

Mosquito mid-gut could be a habitat for several pathogenic organisms. Interaction between these pathogens may influence *Plasmodium* parasites development in the mosquitoes (Yu *et al*., 2022). Experimentally *An. stephensi* mosquitoes infected with maternally inherited

symbiotic *Wolbachia* bacteria were allowed to feed on culture of *P. falciparum* gametocytes. Parasites development was significantly inhibited in *Wolbachia* infected mosquitoes compared to control mosquitoes (Bian *et al*., 2013). Although experimental study should be supported by natural infection, competitive interaction within the mosquitoes may be a promising tool for malaria intervention (Yu *et al*., 2022).

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The present study was conducted in Adama town and the surrounding health facilities of East Shewa zone, Oromia Regional State, Ethiopia. Health facilities of Adama ($8^{o}32'31''$N and $39^{o}16'33''$E), Modjo ($8^{o}26'36''$N and $39^{o}26'36''$E), Awash Melksa ($8^{o}24'25$N and $39^{o}20'02''$E), Olenchiti ($8^{o}38'16''$N and $39^{o}24'19''$E) and Metehara ($8^{o}86'95''$N and $39^{o}92'02''$E) towns were included in the study (Figure 5). Adama town is located at approximately 84Km Southeast of Addis Ababa, the capital city of Ethiopia. Other towns are within 25km radius except Metehara town which is found at 91km East of Adama town.

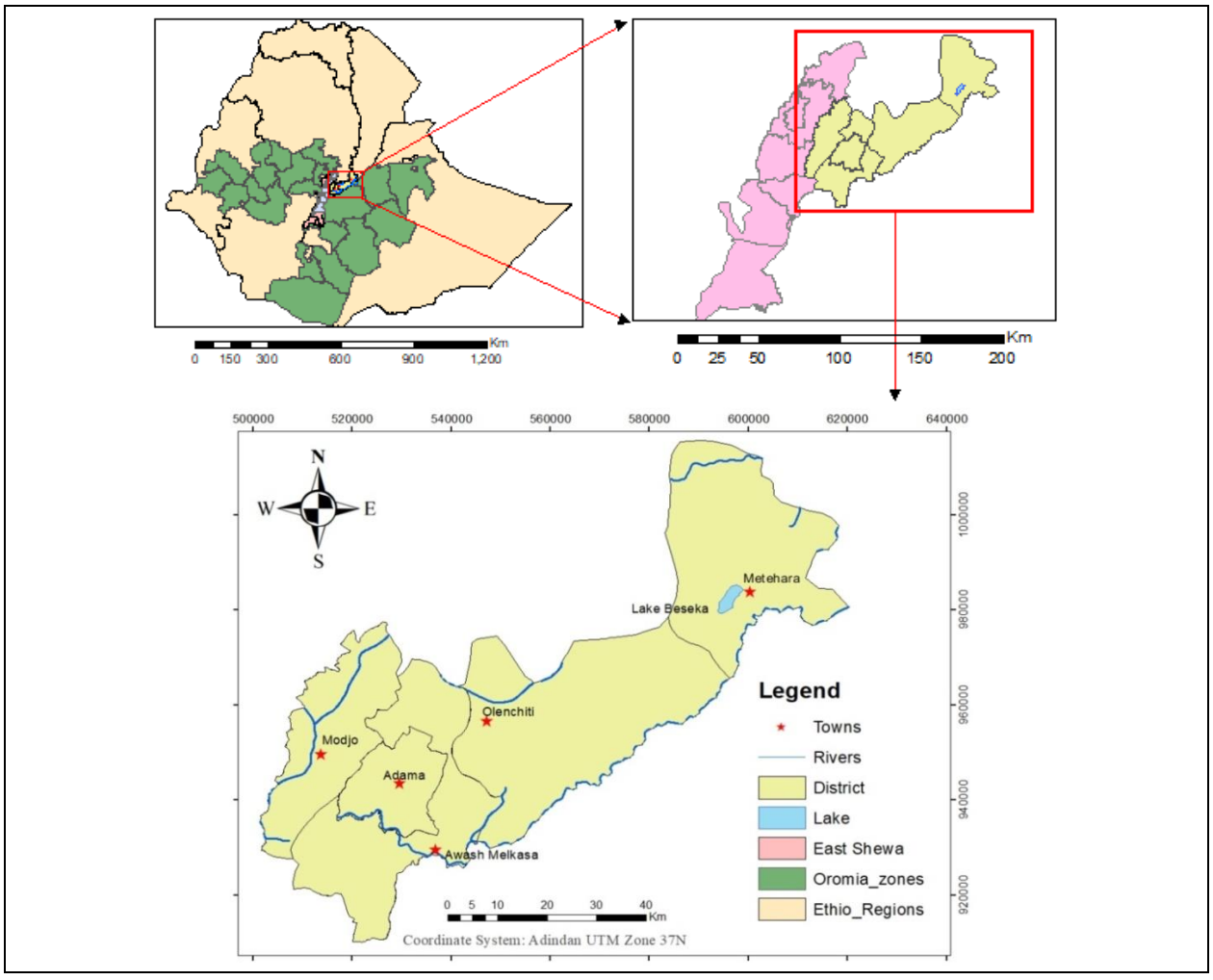


Figure 5. Map of the study areas. Arc GIS version 10.8.

Based on population projection of 2021 made by East Shewa and Adama health offices, the total population of East Shewa zone and Adama town was 2,013,322 among which 49.1% and 50.9% are males and females, respectively. Population of the area mainly engaged in farming, trading, livestock keeping, civil service and non-governmental organizations. There are 285 health posts, 110 clinics, 68 health centers and 8 hospitals in the East Shewa zone and Adama town.

The study area is malaria endemic located in the Great Rift Valley in central Ethiopia. Malaria transmission in the area is seasonal and unstable. Malaria transmission in the area fluctuates from year to year depending on the rainfall patterns. However, major transmission usually occurs from August to December while minor transmission happens from May to June (Golassa & White, 2017). Malaria cases are due to *P. falciparum* and *P. vivax* which co-exist in the area. During major transmission season malaria cases are mainly due to *P. falciparum*; while during dry season malaria cases are dominated by *P. vivax* may be because of relapse infections (Golassa & White, 2017). Mixed infection of both parasites is also common in the area. *An. arabiensis* is the major malaria vector in the area (Adugna *et al*., 2021).

3.2. Study Design

Health facility-based cross-sectional study was conducted at purposively selected health facilities of East Shewa zone from October, 2019 to September, 2021. The health facilities were selected purposively because of their proximity to the insectary and malaria diagnostic center at Adama town to conduct membrane feeding experiments. For DMFA patients were recruited from Adama, Olenchiti, Modjo, Awash Melkasa and Metehara health centers. *Plasmodium falciparum* infected blood samples collected from Awash Melkasa and Modjo health centers were very low, therefore samples collected from Adama, Olenchiti and Metehara health centers were included for molecular study.

3.3. Study Population and Blood Sample Collections

Before the commencement of the study, advocacy visit was conducted to the selected health facilities to inform health centers directors and laboratory technologists about the aims and objectives of the study. At each health facilities after the purpose of the study was explained to patients infected with malaria, consent was obtained. Patients with complicated malaria and those who took anti-malarial drugs within the month were excluded from the study. Pregnant

women and children under five years of age were not involved in DMFA. From those voluntary participants who fulfilled inclusion criteria capillary and venous blood samples (5ml) were collected by experienced laboratory technologists. The blood samples collected were used for microscopic diagnosis, dried blood spot (DBS) preparation for molecular study and membrane feeding experiments.

3.4. Microscopic Diagnosis

Finger-prick blood samples made on the slides were examined for the presence of malaria at health facilities by experienced laboratory technicians according to WHO protocol (WHO, 2010c). Giemsa-stained thick and thin blood smear were employed for the diagnosis of *Plasmodium* parasites. Shortly thereafter, thin films were fixed with methanol for 15-30 seconds. Both thick and thin blood smears were stained with 10% Giemsa solution for 20 minutes. After staining slides were rinsed with water and allowed to air-dry. Following the standard protocol, the stained smears were investigated under high power fields (100X oil immersion) of light microscope to confirm the presence of *Plasmodium* parasites. Thin films were examined to confirm *Plasmodium* species detected on thick film. Thick smears were considered negative when no parasite or gametocyte was detected after examining 100 fields under high power (100X) oil immersion microscopic objective. For quality assurance, each slide was double read for *Plasmodium* species confirmation and parasite and gametocyte densities count by two WHO certified microscopists at Adama malaria research and diagnostic center.

Densities of parasitaemia and gametocytaemia were determined by counting the number of parasites against 200 and 500 white blood cells (WBCs), respectively, on a thick blood film assuming a total WBCs count of 8,000/µl of blood as a standard. Thus, the densities of the parasites and gametocytes were computed by using the following formulae (Nega *et al*., 2015):

$$\text{Parasite density} = \frac{\text{No.of asexual parasite counted x } 8000/\mu l}{200\ \text{WBCs}}$$

$$\text{Gametocyte density} = \frac{\text{No.of gametocyte counted x } 8000/\mu l}{500\ \text{WBCs}}$$

. Parasite and gametocyte densities were determined by averaging the results of the two independent readers.

Parasitaemia was grouped as low (<1000 parasites/ µl blood, moderate (1000-9,999) and high (>10,000 parasites/µl blood) for comparison with *Pfcrt* K76T wild and mutant alleles (Atroosh *et al*., 2012).

3.5. Collection of Blood Samples on Filter Papers

From those patients confirmed for the presence of *P. falciparum* by microscopy four drops of blood (60µl each) were collected on Whatman 3MM ™ filter paper measuring approximately 2.0cm X 5.0cm. The whole blood samples collected on filter paper were allowed to air dry in horizontal position at room temperature for at least four hours away from dust and direct sun light. Each DBS was labeled with patient code and date of collection and kept into small zip-locked plastic bags with 1-2 desiccant sachets or silica gels to protect the specimens from moisture. These zip-locked plastic bags then packed in a larger plastic bag and stored at 2-8^{o}C refrigerator for short time in the field and further stored in deep freezer at -20^{o}C at Aklilu Lemma Institute of Pathobiology (Addis Ababa University) for long period until DNA analysis for molecular study.

3.6. Molecular Analysis

3.6.1. Extraction of *Plasmodium falciparum* DNA

Genomic DNA was extracted from DBS by Chelex-resin extraction method. Briefly, about 3mm-5mm pieces of filter papers which hold approximately 3-5µl of blood were punched out into 1.5ml micro centrifuge tubes. Filter papers were soaked in 1ml of 0.5% Tween® 20 (Sigma Aldrich, USA)/ phosphate buffered saline (PBS) and incubated overnight at 4^{o}C. The mixture was briefly vortexed and centrifuged at 13,000 rpm for 10 minutes. Then, the solution was discarded and filter papers were again soaked in 1ml PBS and kept at 4^{o}C for 30 minutes. After centrifugation, the liquid was discarded and 300µl of 6% Chelex® 100, Molecular Biology Grade Resin (BIO-RAD) in DNase and RNase-free water was added to each tubes. The mixture was heated at 95^{o}C for 12 minutes, opened and briefly vortexed between heating at 3 minutes intervals. Then, centrifugation was conducted at 13,000 rpm for 8 minutes and the supernatant was transferred into new 1.5ml tubes. Again, the supernatant was centrifuged at 13,000 rpm for 1 minute, to ensure complete elimination of Chelex beads. Finally, 250µl supernatant was transferred into 1.5ml tubes and held at -20^{o}C until utilized for molecular analysis.

3.6.2. PCR-Restriction Fragment Length Polymorphism Analysis of *Pfcrt* K76T and *Pfmdr1* N86Y Codons

3.6.2.1. Amplification of the *Pfcrt* Gene

Nested PCR was conducted to amplify *Pfcrt* K76T codon according to the protocol described by Mulenga *et al*. (2021) with some modifications. Briefly, nested 1 PCR was conducted in total reaction volume of 20.0µl consisting of 11.6µl nuclease free water, 0.2µl of each forward and reverse primers, 4.0µl of 5X FIREPol® Master Mix (Solis Biodyne) and 4.0µl of template DNA. Pairs of primers used were displayed in Table 1.

Table 1. Sequences of primers used in nested 1 and nested 2 PCR amplifications of *Pfcrt* and *Pfmdr1* genes

Codon	**Primer**	**Sequence 5'-3'**	**Amplicon size (bp)**	**References**
Pfcrt, K76T	CRT-1F	CCGTTAATAATA AATACACGCAG	537	Djimde *et al*. (2001)
	CRT-1R	CGGATGTTACAAAACTATAGTTACC		
	CRT-2F	TGTGCT CAT GTG TTT AAA CTT	145	
	CRT-2R	CAAAACTATAGTTACCAATTTTG		
Pfmdr1, N86Y	MDR1-1F	TTAAATGTTTACCTGCACAACATAG AAAATT	612	Fontecha *et al*. (2021)
	MDR1-1R	CTCCACAATAACTTGCAACAGTTCT TA		
	MDR1-2F	TGTATGTGCTGTATTATCAGGA	526	
	MDR1-2R	CTCTTCTATAATGGACATGGTA-3'		

Nested 1 and nested 2 PCR conditions and programs were performed according to the conditions listed in Table 2.

Table 2. Optimized nested 1 and nested 2 PCR conditions and programs used to amplify *Pfcrt* K76T codon

Round	PCR conditions	PCR Program
Nested 1 PCR	1st denaturation	94°C for 3 min
	No. of cycles	30
	- Denaturation	94°C 30 sec
	-Annealing	56°C 30 sec
	- Extension	65°C 1sec
	- Final extension	65°C 3 min
Nested 2 PCR	-1st denaturation	95°C for 5 min
	- No. of cycles	40
	- Denaturation	92°C 30 sec
	-Annealing	52°C 30 sec
	- Extension	65°C 30 sec
	- Final extension	65°C 3 min

3.6.2.2. Amplification of *Pfmdr1* Gene

Nested PCR amplification was conducted to identify mutation at codon N86Y by using protocol described by Fontecha *et al*. (2021) with slight modifications. Briefly, nested 1 and nested 2 PCRs were performed in 18.0µl total reaction volume consisting of 11.4µl nuclease free water, 1.0µl of each forward and reverse primers (Table 1), 3.6µl 5X FIREPol Master Mix and 1.0µl DNA template. Nested 1 and nested 2 PCR conditions and programs were performed as displayed in Table 3.

Table 3. Optimized nested 1 and nested 2 PCR conditions and programs used to amplify *Pfmdr1* N86Y codon

Round	PCR conditions	PCR program
Nested 1 PCR	-1st denaturation	95 °C x 3 min
	- No. of cycles	20
	- Denaturation	95 °C x 30 s,
	-Annealing	52 °C x 30 s,
	- Extension	72 °C x 1 min,
	- Final extension	60 °C x 5 min.
Nested 2 PCR	-1st denaturation	95 °C x3 min,
	- no. of cycles	25
	- Denaturation	95 °C x 30 s,
	-Annealing	54 °C x 30 s,
	- Extension	72 °C x 60 s,
	- Final extension	72 °C x 5 min

Nuclease free water (negative control) and *Pf*3D7 (CQ sensitive) and K1 (MRA-159G, CQ resistant) strains of *P. falciparum* (positive controls) were employed along with PCR amplification of both *Pfcrt* K76T and *Pfmdr1* N86Y codons. The strains were kindly provided from Aklilu Lemma Institute of Pathobiology and Ethiopian Public Health Institute (EPHI), respectively.

3.6.2.3. Gel Electrophoresis

Nested 2 PCR products of *Pfcrt* K76T and *Pfmdr1* N86Y codons were resolved, respectively, in 2.0% and 2.5% agarose gel stained with ethidium bromide (EtBr). The 2% and 2.5% agarose gels were prepared, respectively, by adding 6gm and 7.5gm agarose powder in 300ml (30ml 5X TBE buffer and 270ml dH_2O) of 0.5X TBE buffer solution (BIONEER Corp, Korea) (P^H= 8.0). Agarose solution was boiled in microwave for two min and 30 sec until it melts and forms uniform solution. After the solution was cooled 4.0µl a red gel stain (EtBr) was added to it and mixed gently. Then, the solution was poured gently to gel cast tray and

allowed to dry for 20-25 minutes. Once it solidified, the comb was removed and the casted gel was transferred into electrophoresis apparatus (Thermo Scientific). The apparatus was filled with 0.5X TBE buffer solution until it fully covers the gel. Four microliter of DNA ladder (Bionexus) was added to the first well while 5.0µl of nested 2 PCR products were added to the next wells followed by negative and positive controls. Electrophoresis was conducted at 100 volts for 45 minutes. After electrophoresis the gel was removed and DNA bands were visualized under ultra-violet (UV) trans-illuminator (USA) and photographed. DNA band sizes were estimated by comparing with 100bp DNA ladder. The remaining nested 2 PCR products of both *Pfcrt* K76T and *Pfmdr1* N86Y codons were kept at 2-8^{o}C refrigerator until genotyping by restriction fragment length polymorphism (RFLP).

3.6.2.4. Genotyping of *Pfcrt* K76T and *Pfmdr1* N86Y Codons by RFLP

Nested 2 PCR products of both codons were subjected to *Artherobacter protophormiae I* (*APoI*) restriction enzyme (RE) digestion (New England Biolabs). The enzyme recognizes and cuts the segment at the RAATTY/YTTAAR sequence. The enzyme used was a time-saver qualified enzyme which can be incubated overnight with no star activity. Nested 2 PCR products were cleaved without undergoing purification procedures. Digestion was conducted according to the manufacturer instructions (Time-Saver ^{TM} protocol) in a total reaction volume of 20.0µl. Positive controls (*Pf*3D7 and K1) and undigested DNA were included in the reaction. The ingredients were added to 1.5ml Eppendorf tube in the order listed in Table 4. The mixture was allowed for brief centrifugation to mix all the contents. Then, the reaction was incubated in dry bath at 50^{o}C for 15 minutes. After digestion enzyme was inactivated at 80^{o}C for 20 minutes. Two-percent and 2.5% agarose gels were prepared as detailed under gel electrophoresis sub-heading. Digested products of *Pfcrt* K76T and *Pfmdr1* N86Y codons were subjected to electrophoresis at 2.0% and 2.5% respectively. After electrophoresis, gel was taken to UV trans-illuminator and image was captured. Sizes of the bands were estimated by comparing to the 100bp DNA ladder.

Table 4. Restriction enzyme digestion conditions for *Pfcrt* K76T and *Pfmdr1* N86Y codons

Components	Final concentration	Volume per sample
Nuclease free water	-	12.5µl
10X NEBuffer^{TM}r3.1	1X	2.0 µl
Template DNA	unknown	5 µl
APoI (10U/ µl)	5U	0.5 µl
Total volume	-	**20.0 µl**

3.6.3. Amplification of *merozoite surface protein-1* (*msp-1*) gene

Some of the samples which were detected as *P. falciparum* by microscopy were not amplified for *Pfcrt* K76T or *Pfmdr1* N86Y codons. These samples were further tested by nested PCR targeting polymorphic region of *msp-1* (block-2) according to the previously published protocol (File *et al*., 2021). Briefly, in the primary PCR reaction, 20.0µl reaction mixture was composed of 11.2µl nuclease free water, 0.4µl of each forward and reverse primers, 4.0µl 5X FIREPol Master Mix and 4.0µl of DNA template. In the multiplex nested PCR, primer pairs targeting the respective allelic types of *msp-1* (MAD20, K1 and RO33) were used in 25.0µl amplification reaction composed of 16.8µl nuclease free water, 0.4 µl of each forward and reverse primers, 5.0µl 5X FIREPol Master Mix and 2.0µl of DNA template. Both primary and multiplex nested PCRs were conducted as described before by File *et al*. (2021) with 35 and 30 cycling conditions, respectively. *Plasmodium falciparum* 3D7 genomic DNA and nuclease free water were included in each reaction as positive and negative controls, respectively. The amplified products varied between 150 and 250 bps. The segments were then resolved in 2.0% agarose gel stained with EtBr and the bands were observed under UV trans-illuminator and photographed to estimate band size corresponding to 100bp DNA ladder.

3.7. Rearing of *Anopheles arabiensis* in the Insectary

Colonies of *An. arabiensis* mosquitoes (Diptera: Culicidae) used for DMFAs were reared in the insectary at Adama malaria research and diagnostic center. In the insectary microclimates (temperature and humidity) and light conditions were adjusted to mimic natural environment (EPHI, 2017). Temperature and humidity were adjusted using Nikai oil radiator (heater) and

water, respectively. Thus, in the insectary mosquitoes were maintained within temperature and relative humidity range of 26-28^{o}C and 80±10%, respectively. Light is controlled based on 12 hours of light and 12 hours of dark (12L: 12D) photoperiod cycling. Larvae were fed with stick fish food and adults were provided with 10% sucrose solution available as soaked cotton balls kept on mosquito cages. Generally mosquitoes were reared according to the EPHI (2017) guideline. Some of the activities performed in the insectary were shown by appendices 1 and 2.

3.8. Human-to-Mosquito Transmission Experiments

After the patients were confirmed positive for malaria by microscopy at health facilities, voluntary participants were recruited and transported from health centers to mosquito infection laboratory established by our group at Adama insectary center. DMFAs were conducted to assess the infectiousness of study participants to mosquitoes. Glass feeders of equal volume were used for the feeding experiment (Sattabongkot *et al*., 2015). Parafilm membrane was stretched and pressed around the outer rim of the glass feeder. The glass feeder (water-jacketed) has an outer chamber through which water circulates and an inner chamber where blood is poured. Membrane feeder set-up of 20 glass feeders (standard small sized) were connected to each other by plastic tubing, which is also connected to circulatory water bath filled up to 24 liters with water.

Feeding assay was performed according to a protocol developed by Ouédraogo *et al*. (2013). Briefly, about 5ml venous blood samples from study participants was drawn into a heparin-coated tubes for mosquito feeding. Blood samples collected were immediately provided for starved female *An. arabiensis* mosquitoes (2 to 8 days old mosquitoes) without screening blood samples for gametocytes. The mosquitoes were selected using mouth aspirator by placing hand closer to the cage. Forty mosquitoes in water proof cups covered with mesh and secured with rubber band were placed in the feeder. Within 10 minutes after taking blood sample, about 300µl freshly drawn blood was poured into each of the glass feeders. Mosquitoes were allowed to feed in the dark for 30-60 minutes via an artificial membrane attached to a water-jacketed glass feeder maintained at 37^{o}C to prevent premature exflagellation of the microgametocytes. The whole feeding system was covered with black cloth to limit mosquito disturbance hence mimicking natural feeding conditions (Appendix 3).

After blood feeding cups were removed from the feeder system. The feeder system was dismantled and the Parafilm was removed from the feeders. The glass feeders were cleaned first in water with detergent and next washed with cold water and left to air dry over night before re-use (Ouedraogo *et al*., 2013).

After blood feeding mosquitoes were allowed to rest for 30 minutes and then, transferred gently from cups to clean cages. Unfed and partially fed mosquitoes were removed by aspiration into new cup and killed by cold. Fully fed (engorged abdomen) mosquitoes were left in the cages. Each cage was labeled with patient's ID, date of feeding and total number of blood fed mosquitoes (Appendix 4). The mosquitoes were maintained on 10% sucrose solution soaked on cotton pads. To minimize the risk of bacterial and fungal contamination of mosquito population, these pads were changed on daily basis (Ouedraogo *et al*., 2013). These mosquitoes were held at a separate room at the same environmental conditions of the insectary until mid-gut and salivary gland dissections. Mosquito mortality was recorded on a daily basis and conditions were adjusted accordingly.

3.9. Detection of Mosquitoes' Infection and Infectiousness

One week after feeding (7 to 8 days), mosquitoes were dissected in a droplet of 1% mercurochrome solution (oocyst absorbs the dye differentially from the rest of mid-gut tissues) under a dissecting microscope (VWR®) and examined for the presence of oocyst. The protocol for mid-gut dissection was adopted from Ouedraogo *et al*. (2013). Briefly, lives blood fed mosquitoes were immobilized by placing them into a freezer for 10 minutes. Then, mosquitoes were anesthetized by transferring to petri-dishes lined with wet paper towels to avoid drying. After immobilization, each mosquito was placed on a microscope slide and held with one wing, while the legs were removed turn by turn and finally the wing was cut off to prevent accidental escape of mosquitoes. The thorax was held with one needle and the tip (last two segments) of the abdomen was slightly pulled away by forceps. Malpigean tubules and ovaries were removed from the mid-guts and then, the mid-guts were stained for 10 minutes with 1% mercurochrome solution (0.5g mercurochrome in 50ml distilled water) on a glass slide. Mid-gut oocysts were examined and counted using light microscope, under 20X magnification. After mid-gut dissection three-forth (75%) of mosquitoes were maintained for salivary gland dissection on 14-15 days post-infection (dpi). Salivary glands from each

mosquito were mounted on glass slide covered with cover-slip in a drop of PBS. Then, salivary glands were disrupted by applying pressure and observed under 40X light microscope. The number of sporozoites was counted and recorded for each mosquito. For each of the infection session, rate of infection and intensity of infection (both for oocysts and sporozoites) were determined. Oocyst and sporozoite infection rates were calculated as summarized by the following formulae:

$$\text{Oocyst infection rate} = \frac{\text{No.of mosquitoes carrying oocysts}}{\text{No. of dissected mosquitoes}} \text{X100}$$

$$\text{Sporozoite infection rate} = \frac{\text{No.of mosquitoes carrying sporozoites}}{\text{No. of dissected mosquitoes}} \text{X100}$$

Images of mid-gut and salivary glands were taken by Pylon Viewer Camera Software Version 4.0 installed on desktop and connected to light microscope (EURO Star III Plus).

3.10. Data Management and Analyses

Statistical analyses were conducted using statistical packages for social sciences (SPSS) and GraphPad Prism softwares version 26 and 8.0, respectively. Chi-square and Fischer Exact tests were used as appropriate to evaluate associations between different variables. Association between asexual and gametocyte densities and age groups was analyzed using non-parametric Kruskal-Wallis test. Linear regression was used to evaluate the association between mosquito infections (oocyst and/or sporozoite infected mosquitoes) and parasitaemia and gametocytaemia. Linear regression was also used to test correlation between mean oocyst and sporozoite loads. A P-value < 0.05 was considered as statistical significance in all the analyses.

3.11. Ethical Approval and Consent to Participate

This study was conducted after ethical clearance was obtained from research and ethical committee of Adama Science and Technology University (Certificate reference No. RECSoANS/BIO/02/2019) (Appendix 5). Permission letter was also obtained from Oromia Regional State Health Bureau, East Shewa zone, Adama town and health offices of the respective study sites. Informed consent was obtained from patients (Appendix 6) and/or their parents or legal guardians in case of children under 18 years (Appendix 7). Study volunteers

were given the right to refuse to participate in the study and to withdraw at any time during the study. Confidentiality of the study participants was assured by using code number instead of their names to protect their identity. Patients confirmed positive for malaria infection were treated according to the national treatment guidelines (MoH, 2017).

CHAPTER 4

4. RESULTS

4.1. Prevalence of *Pfcrt* 76T and *Pfmdr1* N86 Genotypes in Malaria Infected Patients

4.1.1. Socio-demographic and Parasitological Characteristics of Study Population

Socio-demographic and parasitological characteristics of the population for which samples were successfully analyzed for *Pfcrt* K76T (83) and *Pfmdr1* N86Y (80) codons were displayed in Table 5 and Appendix 8. The ages of the patients were between 1 and 68 with mean (SD) age of 23 (±14.7) years. Majority (70%) of the study participants were older than 15 years of age. Sixty-five and thirty-five percent of the study participants were males and females, respectively. Parasite densities ranged from 79 to 157,538 parasites/µl of blood, with a parasitaemia geometric mean of 5366 or median 6579 asexual parasites/µl.

Table 5. Socio-demographic characteristics and parasitaemia level of patients recruited for anti-malarial resistance study (N = 83) from Adama, Olenchiti and Metehara health centers during October, 2019 to September, 2021

Variables	Values n (%)
Sex	
Males	54 (65)
Female	29 (35)
Age (Years)	
Mean (SD)	23±14.7
Range (IQR)	1-68 (13-30)
0-4	7 (8.4)
5-14	18 (21.7)
15-30	37 (44.6)
>30	21 (25.3)
Parasitaemia level	
Geometric mean parasite density (SD)	5366 (25,532.89)
Range of parasite density (IQR)	79-157,538 (1902.5-20,700)
<1000 parasites/µl	15 (18.1)
1000-9,999 parasites/µl	38 (45.8)
>10,000 parasites/µl	30 (36.1)

IQR- interquartile range, SD- standard deviation

The mean parasite density in the present of the three study areas ranged from 9870.95/µl to 15089.22/µl, but there was no statistically significant difference ($\chi 2$ = 162.75; P = 0.469) (Figure 6).

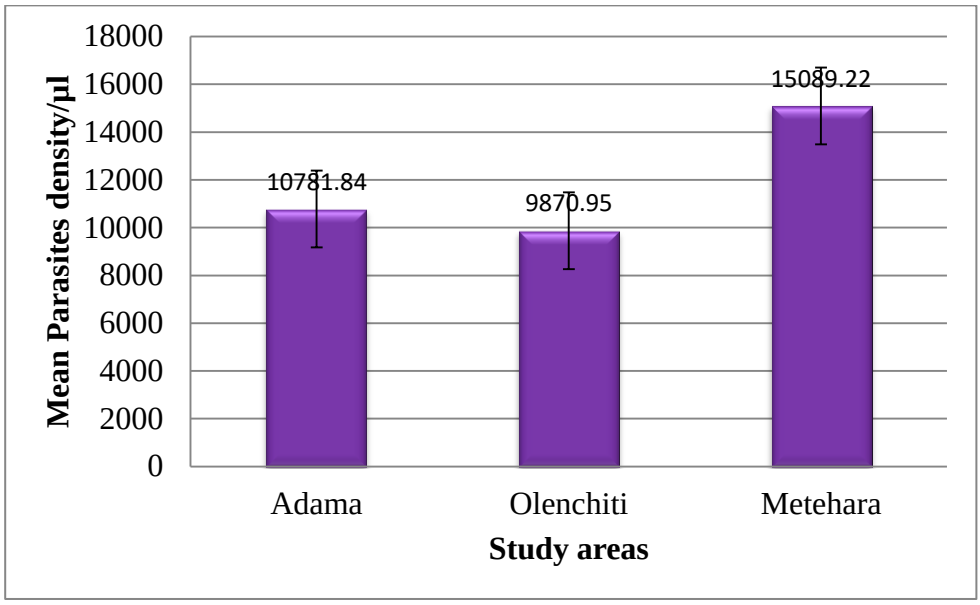


Figure 6. Mean parasites density by study areas. Bars indicate standard error of the mean.

During the study period blood samples were collected from 711 (481 males and 230 females) self-presenting febrile patients. Out of 711 blood samples, 121 (17.0%) of them were microscopically confirmed for *P. falciparum* isolates. Eighty-three (68.6%) of these positive samples were successfully amplified by nested PCR for *Pfcrt* K76T codon while amplification for 38 samples was unsuccessful. From those samples amplified for *Pfcrt* K76T codon, 80 samples were also successfully amplified for *Pfmdr1* N86Y codon. Representative gel images of nested 2 PCR products of both codons resolved in 2.0% and 2.5%, respectively, were shown in Appendix 9.

4.1.2. RFLP Analysis of *Pfcrt* K76T and *Pfmdr1* N86Y Codons

Nested 2 PCR products of the two codons were subjected to digestion by *APoI* RE. Digestion of PCR products carrying *Pfcrt* K76 alleles (wild-allele) resulted in 100 and 45 bps, but remained uncut in the presence of 76T mutant allele (hence a single band of 145bp) while digestion of those products harboring *Pfmdr1* N86 alleles produced 179 and 239bps (representing wild alleles) (Appendix 10).

Of eighty-three *P. falciparum* isolates amplified for *Pfcrt* K76T codon, majority of them carried 76T allele (76, 91.6%) while very few samples (7, 8.4%) carried K76 alleles (Figure 6). When distributed across the study areas, the prevalence of 76T mutant allele was 84.2% (16 of 19), 92.7% (38 of 41) and 95.7% (22 of 23), at Olenchiti, Metehara and Adama, respectively. However, the difference was not statistically significant ($\chi 2 = 1.895$; P = 0.388). On the other hand the prevalence of K76 was 4.3% (1 of 23), 7.3% (3 of 41) and 15.8% (3 of 19) and at Adama, Metehara and Olenchiti, respectively.

Most of these alleles (K76) were detected in *P. falciparum* isolates with moderate parasitaemia level (1000-9,999 parasites/µl). Parasite isolates carrying 76T mutant alleles had higher mean parasite density of 15,420 parasites/µl while those parasites harboring K76 wild alleles had a mean parasite density of 7623.14 parasites/µl. However, no significant association was found between type of allele and parasite density ($\chi 2 = 76.53$; P = 0.620).

Among eighty nested 2 PCR products of *Pfmdr1* N86Y analyzed with the same RE (*APoI*), all of them (100%) were N86 wild-type allele; no single 86Y mutant allele was detected in the current study (Figure 7).

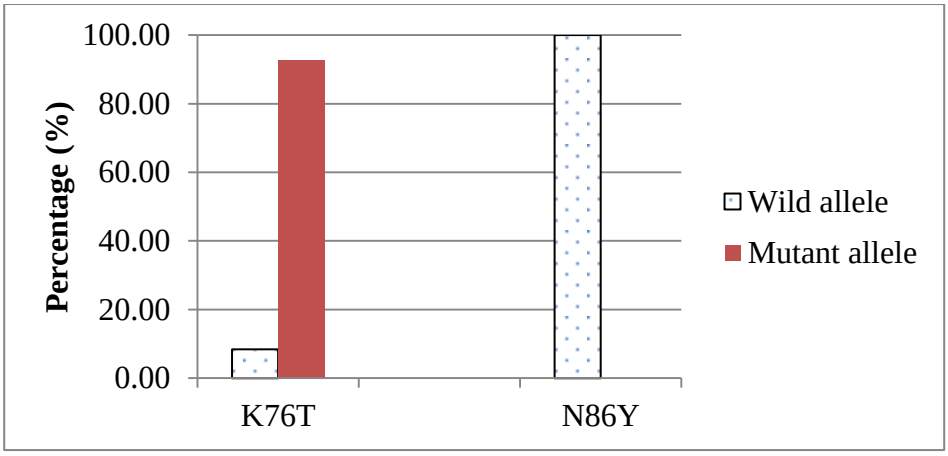


Figure 7. Prevalence of wild and mutant alleles for *Pfcrt* K76T and *Pfmdr1* N86Y codons among *P. falciparum* isolates from Adama, Olenchiti and Metehara study areas during October 2019 to September, 2021.

4.1.2.1. Proportions of *Pfcrt* 76T Alleles According to Gender and Age

Among 83 *P. falciparum* isolates successfully genotyped for K76T codon, 54 and 29 were males and females, respectively. In the present study the number of males was greater than

females and they contributed for higher proportion (64.5%) of *Pfcrt* 76T mutant allele. However, the difference was not statistically significant among gender (P = 0.712). Proportion of this allele among gender across study areas was shown in Figure 8.

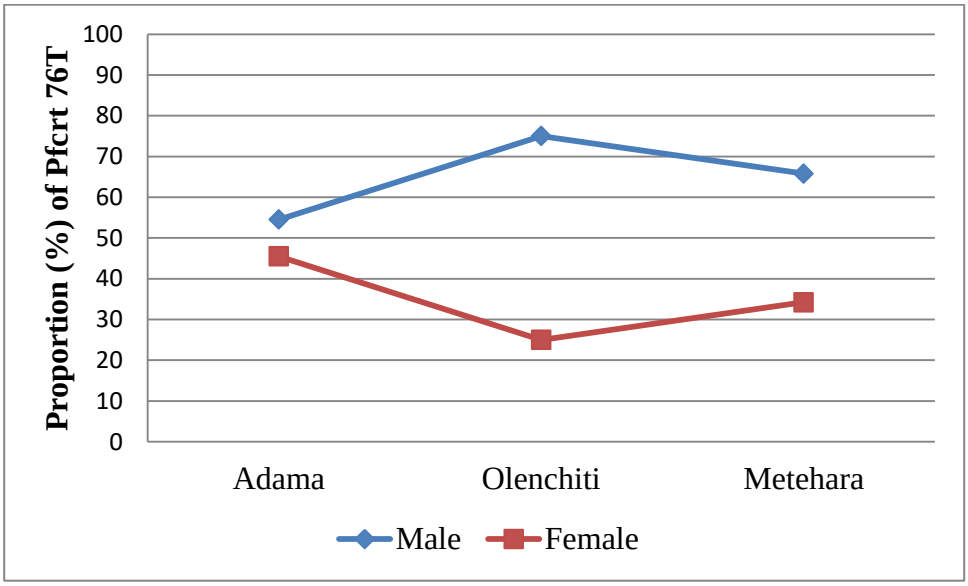


Figure 8. Proportion of *Pfcrt* 76T allele among gender per study areas during October 2019 to September, 2021.

On the other hand, prevalence of this allele (76T) was assessed across age group at Adama, Olenchiti and Metehara areas. High prevalence of 76T allele was detected among age groups of 15-30 years with proportions of 56.3%, 45.5% and 42.1 % at Olenchiti, Adama and Metehara areas, respectively (Figure 9). However, the difference was not statistically significant among age groups (Fischer Exact test, P = 0.69).

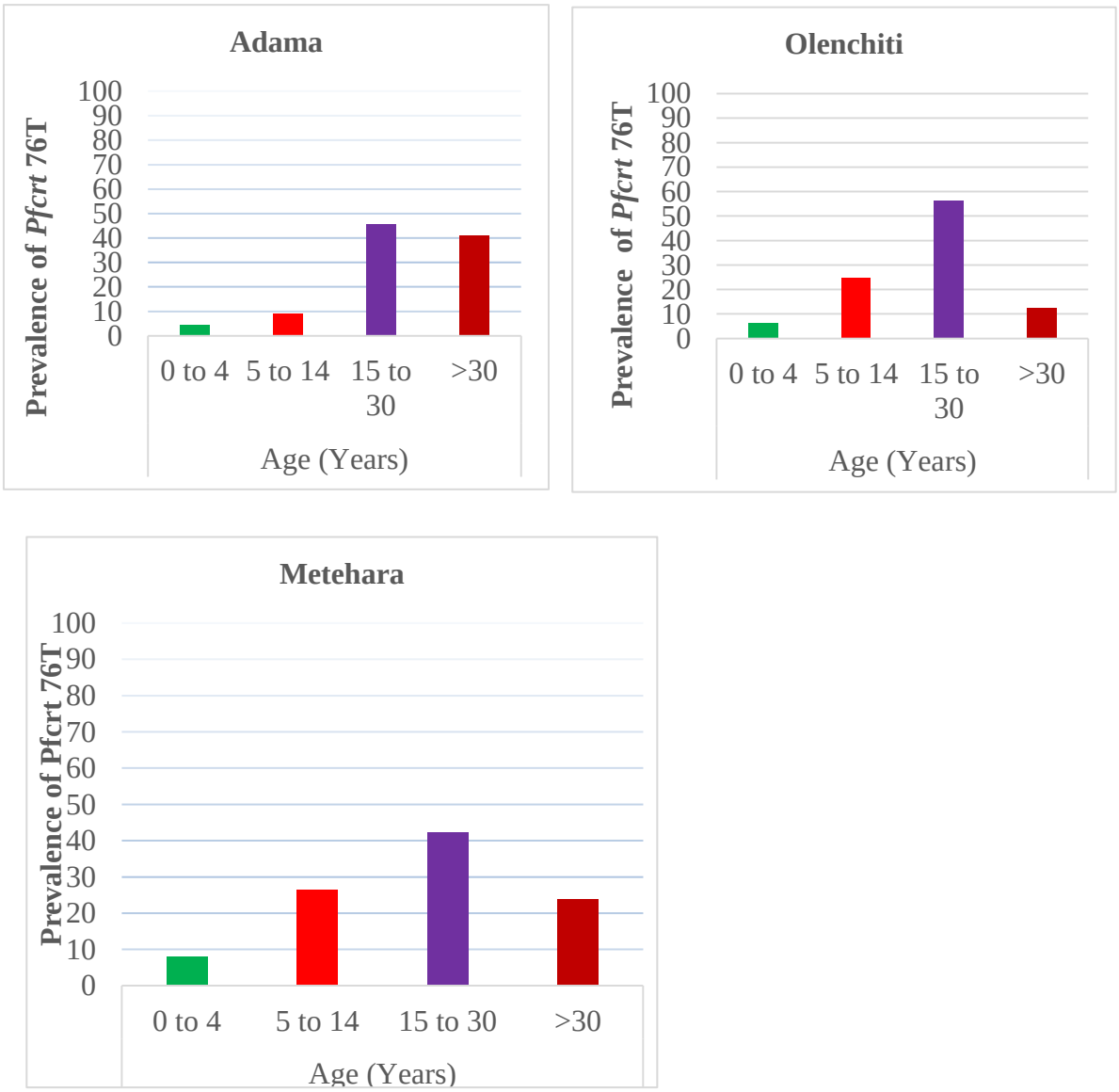


Figure 9. Proportions of *Pfcrt* 76T allele among age groups per study areas during October, 2019 to September, 2021.

4.2. Amplification of *Pfmsp1* for Samples not amplified for *Pfcrt* K76T and *Pfmdr*1 N86Y Codons

Nested PCR amplification for *Pfmsp1* allelic families of polymorphic region (Block-2) was conducted on 38 samples which were confirmed microscopically for *P. falciparum* isolates but not amplified by PCR particularly for *Pfcrt* K76T codon. Majority (71.1%) of those samples were from Metehara health center followed by those of Adama (21.0%) and Olenchiti (7.9%)

health centers. Among 38 of those samples, 26 (68.4%) were successfully amplified while 12 (31.6%) samples were not amplified for the gene under report.

4.3. Infectivity of Symptomatic Malaria Patients to *Anopheles arabiensis* Mosquitoes

4.3.1. Characteristics of Study Participants

Feeding assays were successfully conducted for 63 patients of whom 45 (71.4%) were males and 35 (55.6%) were ever married (married, divorced and separated). The majority (66.7%) of the patients were recruited from Adama town health centers and Adama malaria diagnostic center while the remaining 33.3% were from health centers outside of Adama town. Detailed of descriptive statistics of the study participants and parasite stages were displayed in Table 6 and Appendix 11.

Table 6. Demographic characteristics and microscopic diagnosis malaria among study participants involved in DMFAs from East Shewa zone, Oromia Regional State, Ethiopia during October, 2019 to September, 2021.

Variable	Category	Frequency (%) or median (range)
Sex	Male	45 (71.4)
	Female	18 (28.6)
Age	≤24	22 (34.9)
	25-44	25 (39.7)
	≥45	16(25.4)
Marital status	Ever married	35 (55.6(
	Never married	28 (44.4)
Residence	Adama town	42 (66.7)
	Out of Adama town	21 (33.3)
Plasmodium species	*P. vivax*	45 (71.4)
	P. falciparum	15 (23.8)
	Mixed (*P. vivax* and *P. falciparum*)	3 (4.8)
Parasite stages	Asexual parasite density/µl	4098 (111-19,582)
	Gametocyte density/µl	71 (0-2785)
Gametocyte density	Urban	160* (146-2395)
	Rural	199* (48-2785)

*Mean values

Asexual parasite densities of age group ≤24 (median of 6681parasites/µl, IQR: 2104-8316) and 25-44 (median of 5592 parasites/µl, IQR: 1308-8238) were closer to each other but significantly lower in age group ≥45 (median of 2190 parasites/µl, IQR: 622-4233) (Kruskal Wallis test, P = 0.028). Age group 25-44 tended to have higher median gametocyte density (median of 97.5 gametocytes/µl, IQR: 66.5-213.75) than age group ≤24 (median of 91.5 gametocytes/µl, IQR: 8.5-191.25) and ≥45 (median of 63.5 gametocytes/µl, IQR: 49-79.5). However, no significant difference gametocyte density was found across categories of age groups (Kruskal Wallis test, P = 0.054) (Figure 10). Age group 25-44 was found more infectious to mosquitoes compared to other age groups; although infectiousness was not

significantly different among age groups (P = 0.057) (Table 7). Gametocyte densities were higher among *P. vivax* infected and female patients (Figure 11).

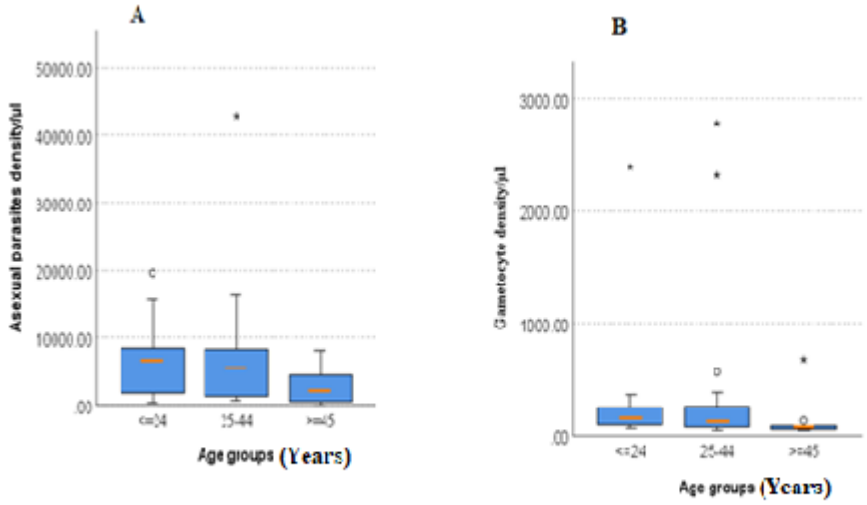


Figure 10. Asexual stage of parasites (A) and gametocytes density (B) distributions among age groups of study participants involved in DMFA at East Shewa Zone, Oromia Regional State, Ethiopia during October, 2019 to September, 2021.

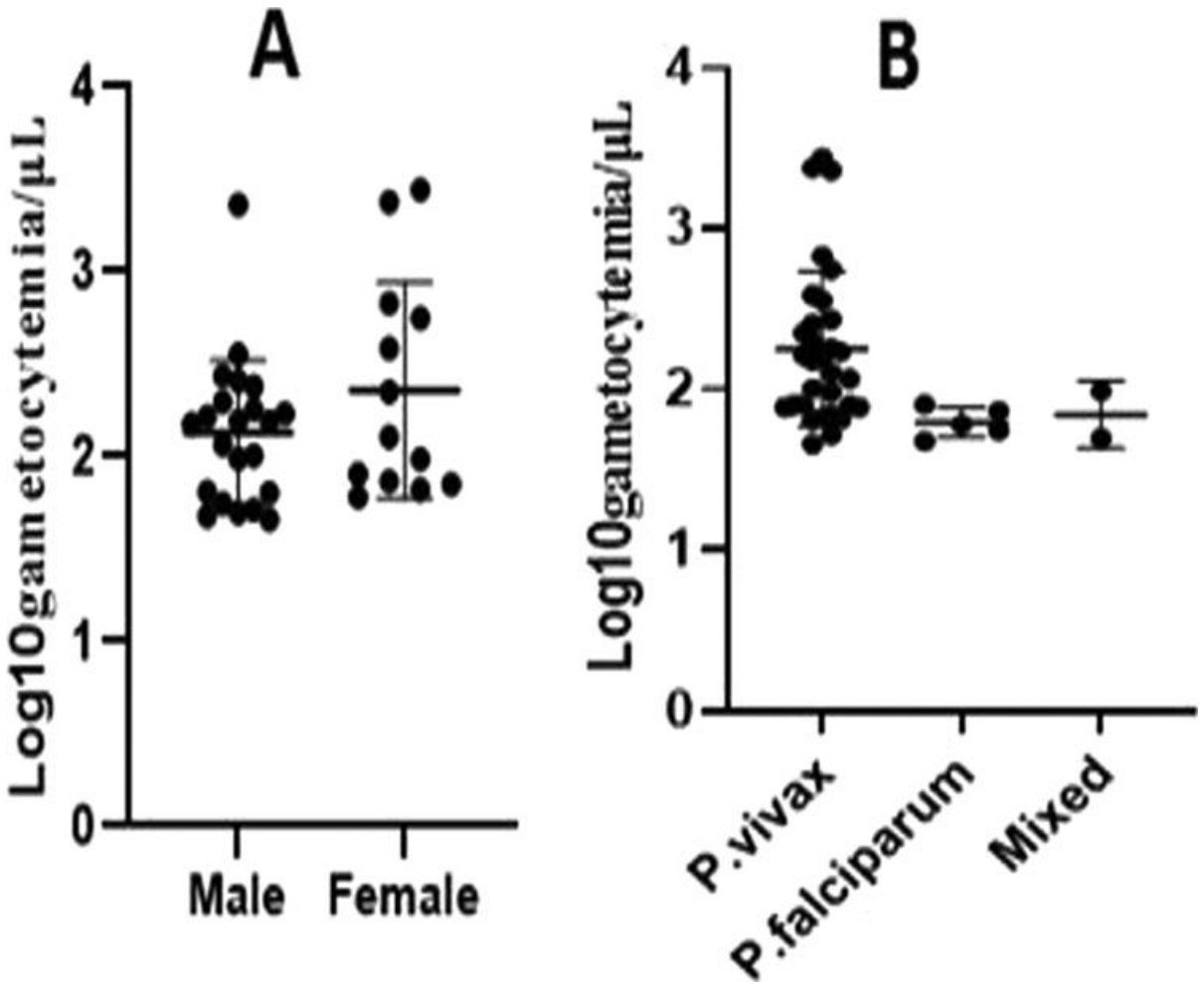


Figure 11. Gametocytes densities (mean values + standard deviation) by sex of participants (A) and *Plasmodium* species (B).

4.3.2. Infectiousness of the Study Participants

Out of 63 DMFA experiments conducted, 43 (68.3%) of the patients infected at least one mosquito while the remaining 20 (31.7%) were not infectious to mosquitoes (mosquitoes failed to produce oocyst). Among *P. vivax*-infected patients, a higher proportion (77.8%) of patients were infectious to mosquitoes whereas from *P. falciparum* infected patients, below half (46.7%) were found infectious to mosquitoes. Of all individuals who were gametocyte carriers 84.8% (39 of 46) infected at least one mosquito. All *P. falciparum* gametocyte carriers (100%, 5 of 5) could infect ≥1mosquitoes, while 15.8% (6 of 38) and 50% (1 of 2) of *P. vivax* and mixed cases, respectively, with microscopy detectable gametocytes failed to infect mosquitoes. In contrary, 23.5% (4 of 17) of gametocyte negative cases (two of *P. vivax* and

two of *P. falciparum*) could infect mosquitoes. Females, rural residents and *P. vivax* infected patients were more infectious to mosquitoes than males, urban residents and *P. falciparum* infected patients, respectively (Table 7).

Table 7. Mosquito infectiousness of the study participants in East Shewa zone, Oromia Regional State, Ethiopia during October, 2019 to September, 2021

Variable	Category	n (%) having at least one mosquito with oocyst(s)		
		No	Yes	χ2
Sex	Male	18 (40.0)	27 (60.0)	0.036*
	Female	2 (11.1)	16 (88.9)	
Age	≤24	8 (36.4)	14 (63.6)	
	25-44	4 (16.0)	21 (84.0)	0.057
	≥45	8 (50.0)	8 (50.0)	
Marital status	Never married	11 (39.3)	17 (60.7)	
	Ever married	9 (25.7)	26 (74.3)	0.250
Residence	Urban	17 (40.5)	25 (59.5)	
	Rural	3 (14.3)	18 (85.7)	0.046*
Plasmodium	*P. vivax*	10 (22.2)	35 (77.8)	
species	*P. falciparum*	8 (53.3)	7 (46.5)	0.03*
	Mixed	2 (66.7)	1 (33.3)	

*Fisher exact test; n, number

4.3.3. Prevalence of Infected and Infectious Mosquitoes Following Direct Membrane Feeding Assay

To determine prevalence of infected and infectious mosquitoes mid-gut and salivary gland dissections, respectively, were carried out. For every DMFA, on average 175 (40-550) female adult mosquitoes were exposed to *Plasmodium* infected blood. Overall, mosquito feeding rate was 61.8% (6,795/11,000 ranging from 18 to 330 mosquitoes per assay) of mosquitoes. Among mosquitoes used for DMFAs, 62.1% (5,230/8,420), 64.0% (1,369/2,140) and 44.5% (196/440) of mosquitoes were engorged after they were exposed to blood samples from *P. vivax*, *P. falciparum* and mixed species infected patients, respectively (Table 8 and Appendix 12).

4.3.3.1. Mid-gut Infection Rates of Mosquitoes

On average, 23 (ranges, 5-60) mosquitoes per assay were dissected 7-8 dpi for oocyst detection. Appendix 13 shows an example of *An. Arabiensis* mosquito mid-gut dissected for *P. vivax* oocysts detection post-7 days of infection.

Among mid-gut dissected mosquitoes, 21.6% (Table 8) were infected with oocysts with geometric mean of nearly 8 oocysts per mosquito (ranging from 1 to 285 oocysts/mid-gut). The median oocyst load per assay was 42, ranging from 4 up to 2,625 per study participant (Appendix 14).

Table 8. Characteristics details of mosquitoes involved in DMFAs in East Shewa zone, Oromia Regional State, Ethiopia during October, 2019 to September, 2021

Characteristics		**Value for**			
		P. vivax	***P. falciparum***	**Mixed species**	**Total**
No. of:	Exposed mosquitoes	8,420	2,140	440	11,000
	Fed mosquitoes	5,230	1,369	196	6,795
Age (days) range of exposed mosquitoes (mean ± SD)		2-8 (4.49± 1.40)			NA
Starvation period (h) ± SD (range)		11:45±3.85 (4-20)			NA
% of mid-gut oocyst infected mosquitoes		30.3 (271/895)	6.7 (29/431)	2.8 (2/72)	21.6 (302/1,398)

NA - not applicable; h - hour; SD - standard deviation

In this study, 32.7% (292/892) and 8.5% (10 of 117) of mosquitoes were infected with oocysts from microscopy positive and negative gametocyte blood samples, respectively. Among all mid-gut analyzed mosquitoes highest (30.3%) proportions were infected with *P. vivax*

followed by *P. falciparum* (6.7%) and mixed infections (2.8%). Similarly, relatively higher oocyst loads were detected in mosquitoes fed on blood of *P. vivax* infected patients (Figure 12).

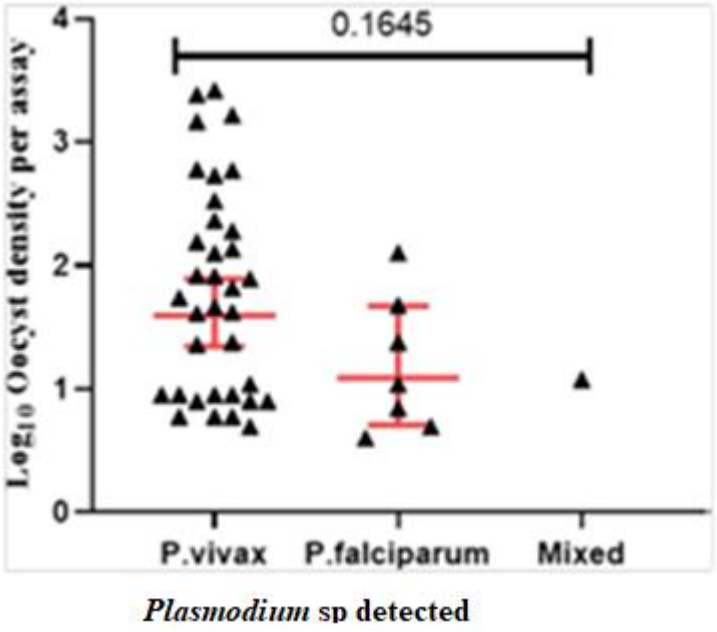


Figure 12.Oocysts densities (mean values [Log10 transformed] + standard deviation) of mid-guts per infectious individuals based on *Plasmodium* species.

Though statistically significance and positive correlation exists between oocyst infection rates of mosquitoes and parasitaemia (R^2 = 0.099, P = 0.04) and gametocytaemia (R^2 = 0.41, P = 0.0001), the correlation was weak. Oocyst loads were significantly correlated with parasitaemia (R^2 = 0.2118, P = 0.002) and gametocytaemia (R^2 = 0.5554, P = 0.0001) (Appendix 15). We also detected significant and moderate correlation between oocyst load per assay and the proportion of infected mosquitoes (R^2 = 0.672, P < 0.0001) (Figure 13).

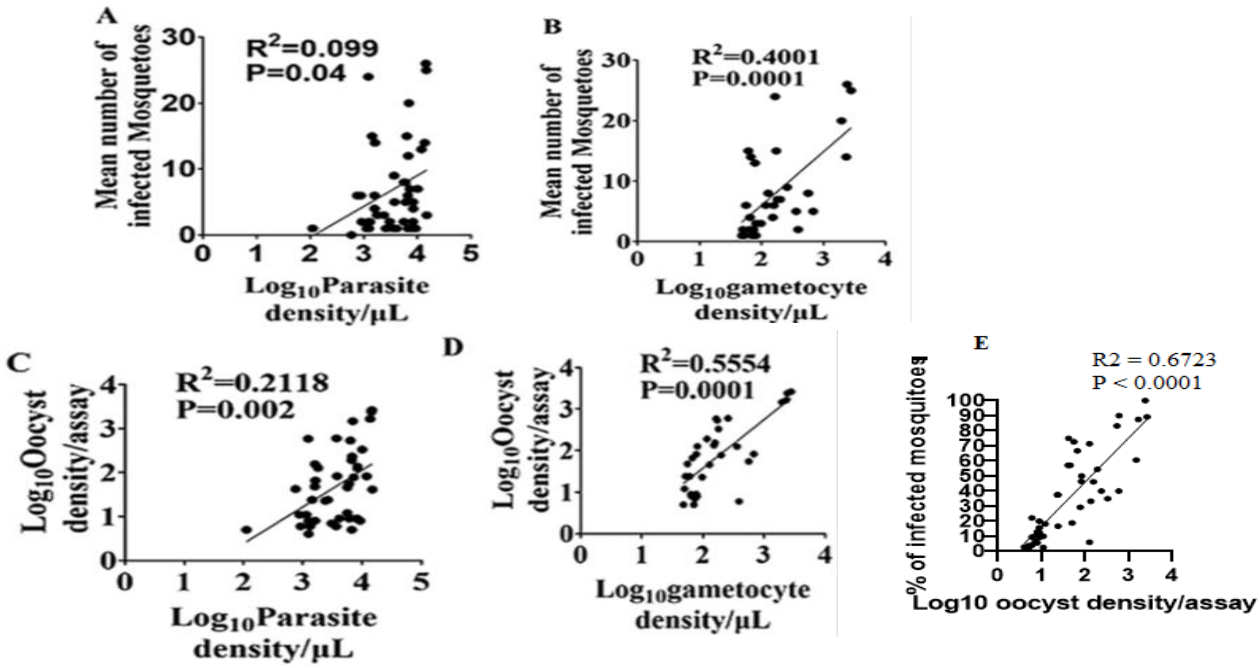


Figure 13. Relationship between infected mosquitoes and *Plasmodium* species parasitaemia, gametocytaemia and oocyst densities of each DMFA conducted in East Shewa zone, Oromia Regional State, Ethiopia during October, 2019 to September, 2021.

4.3.3.2. Salivary Gland Dissection for Sporozoite Infection Rates

To calculate sporozoite infection rates, salivary gland dissection was performed on mosquitoes fed on blood samples from 28 (65.1%, 28 of 43) and 2 (10%, 2 of 20) infectious and non-infectious participants, respectively. Nearly, 57% (17 of 30) of the participants were found to infect mosquitoes with sporozoites. All sporozoites were detected in salivary glands of mosquitoes fed on blood from *P. vivax* infected participants. Among the randomly selected assays with oocyst infected mosquitoes, 60.7% possessed sporozoite infected mosquitoes. Four percent (284 of 6795) of mosquitoes were dissected 14 dpi for salivary glands to detect sporozoites infections. Appendix 16 shows images of salivary gland dissected for mosquitoes infected with *P. vivax* sporozoites.

From 30 participants, sporozoite infection rate was 20.4% (58 of 284 mosquitoes) with mean of 320 (range of 56 to 1337) sporozoite loads per assay. Each salivary gland was infected with a median of 92 (ranged of 56 to 1253) sporozoites. Positive and statistical significant correlation was observed between mosquitoes infected with sporozoites and gametocytaemia

(R^2 = 0.51, P = 0.0001), mean oocyst and sporozoite infected mosquitoes (R^2 = 0.478, P = 0.0001), mean of sporozoite infected mosquitoes and mean number of oocyst per assay (R^2 = 0.568, P = 0.0001) and sporozoites and oocyst loads (R^2 = 0.376, P = 0.0001). However, no significant correlation was observed between proportions of sporozoite infected mosquitoes and parasite densities (R^2 = 0.114, P = 0.07) (Figure 14).

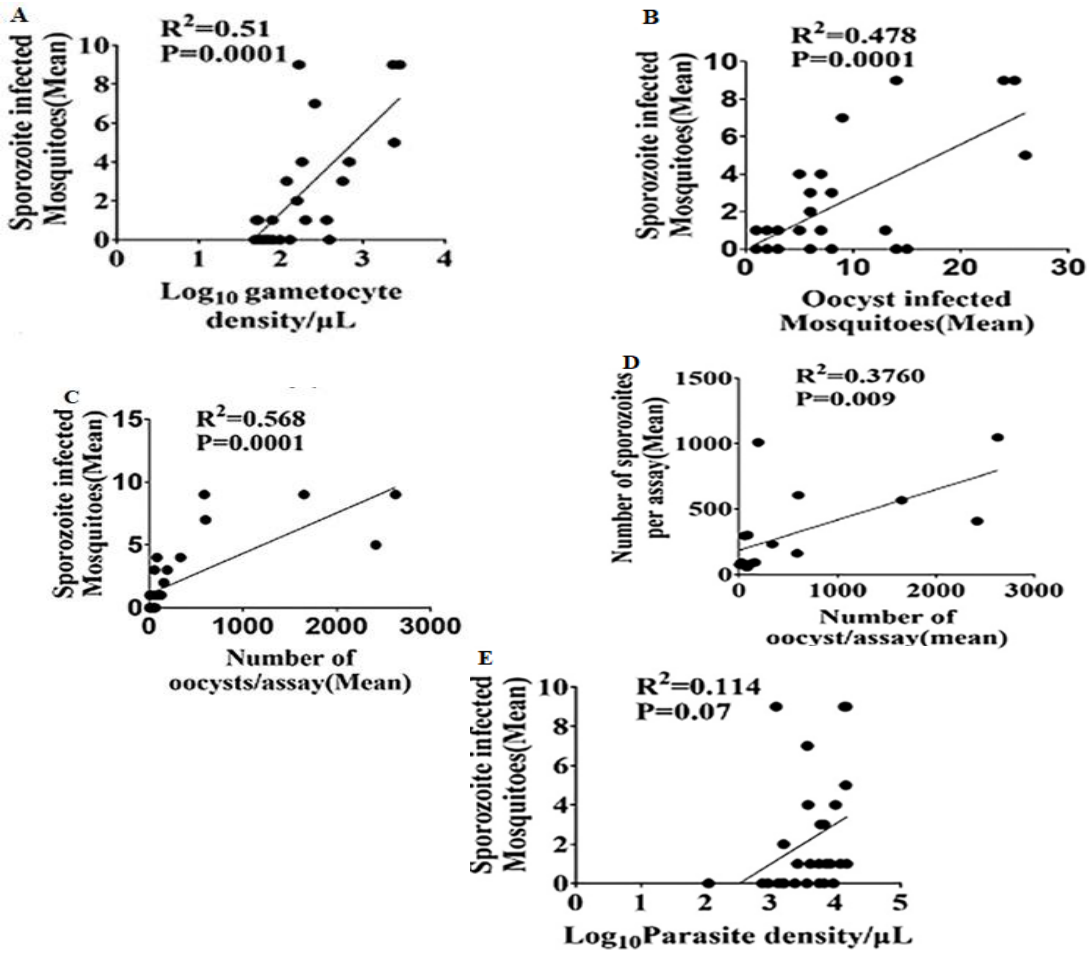


Figure 14. Relationship between sporozoite infection and gametocyte, parasite and oocyst densities of each DMFA conducted in East Shewa zone, Oromia Regional State, Ethiopia during October, 2019 to September, 2021.

CHAPTER 5

5. DISCUSSION

5.1. Prevalence of *Pfcrt* 76T and *Pfmdr1* N86 Genotypes in Malaria Infected Patients

Currently, one of the most hurdles to control and/or eliminate malaria globally is due to emerging and expansion of anti-malarial drug resistant *Plasmodium* parasites. Particularly, *P. falciparum* inherited resistance to every class of anti-malarial drugs including ART (Roux *et al*., 2021). Molecular marker-based surveillance is useful as the markers are good predictors of emerging or current status of resistance to anti-malarial drugs. For example, a recent review on molecular markers surveillance indicates that after withdrawal of CQ a decline in prevalence of *Pfcrt* K76T and *Pfmdr1* N86Y mutations were reported in ten SSA countries (Njiro *et al*., 2022).

In the current study high prevalence of *Pfcrt* 76T mutant allele (91.6%) was reported from the study areas. However, the prevalence of this allele was not statistically different across the study areas. Conversely, low prevalence (8.4%) of *Pfcrt* K76 wild-type allele was detected in *P. falciparum* isolates. This finding does not support a previous high prevalence (10-fold of the current prevalence) of this wild-type allele from Southern and Eastern parts of Ethiopia (Mekonnen *et al*., 2014). The current high prevalence of the mutant allele in the present study areas is in line with previous studies conducted in different parts of the country (Mula *et al*., 2011; Heuchert *et al*., 2015; Golassa *et al*., 2015; Hailemeskel *et al*., 2019). This continuous high prevalence of the mutant allele in Ethiopia may be associated with partial withdrawal of CQ as it is currently used as first-line treatment for *P. vivax* which accounts approximately for 40% of malaria cases in Ethiopia (Alelign and Dejene, 2016). Persistently high prevalence of vivax malaria in the form of relapse during dry season (Golassa & White, 2017) may expose *P. falciparum* to CQ pressure which rises in prevalence in the next malaria transmission season. The current high prevalence of the mutant allele may be also associated with the dominance of *P. vivax* over *P. falciparum* (Golassa & White, 2017; Hailemeskel *et al*., 2019) where there is possibility of misdiagnosing *P. falciparum* as *P. vivax* and treating with CQ (Jaleta *et al*., 2020).

Circulations of parasites with high frequencies of this mutant alleles may also be attributed to the co-existence of the two parasites as mixed infections (Ketema *et al*., 2021). Misdiagnosis of malaria such as mixed infections (Golassa *et al*., 2014) as *P. vivax* and treating patients with CQ was usually observed during the study period and this experience may expose *P. falciparum* to indirect CQ pressure selecting for the mutant genotypes. For instance, among 36 mixed infections confirmed microscopically by WHO certified microscopists (at Adama malaria diagnostic center), 83.3% (30/36) and 16.7% (6/36) (unpublished data) were reported as *P. vivax* and *P. falciparum* infections, respectively, from the study areas. Cryptic CQR *P. falciparum* parasites occurred as a result of mixed infections misdiagnosed as *P. vivax* and treated with CQ (Mayxay *et al*., 2001).

At the early implementation of AL in Ethiopia, study conducted by Shunk *et al*. (2006) from Southern Ethiopia showed 100% prevalence of 76T allele in parasites isolates. Almost ten years later, since the implementation of AL, the same level of 76T allele was reported from Adama (Golassa *et al*., 2014) one of the present study areas. Although parasites with wild-type allele (K76) recovered at frequencies of 8.4% in the present study, in this low malaria endemic area (MOH, 2020), parasites isolates with mutant allele may still circulate at high frequencies between human host and *Anopheles* mosquito vectors may be for the reasons described below.

Rate of sexual recombination of parasite genotype is low in low malaria endemic settings; which might result in the more resistant genotypes to be established in the population (Laufer *et al*., 2004). Thus, malaria transmission involving multiple genotypes is uncommon in low transmission areas. This might create conducive conditions for the sustained transmission of parasites carrying mutant genotype in the current study areas. Conversely, in high malaria transmission areas, where a large proportion of infections are not treated, within host competition between drug sensitive and resistant parasites (Bushman *et al*., 2018), higher level of acquired immunity and higher rate of recombination in mosquito vectors may slow the spread of drug resistant parasites (Yobi *et al*., 2020; Mulenga *et al*., 2021). As an evidence significantly higher prevalence of 76T and K76 alleles were detected respectively in low and high malaria endemic settings of Ethiopia (Hailemeskel *et al*., 2019); showing that CQ-sensitive parasites slowly return in low transmission areas.

The current high prevalence of mutant allele (76T) is in line with reports from Yemen (Al-Mekhlafi *et al*., 2011), Malaysia (Atrosh *et al*., 2012), India (Das *et al*., 2017), Nigeria (Adam *et al*., 2021), Bangladesh (Johora *et al*., 2021) and Angola (Ebel *et al*., 2021) where CQ continues for the treatment of vivax malaria. Conversely, very low prevalence of this mutant allele was documented in SSA countries such as Malawi (Frosch *et al*., 2014), Zambia (Mwanza *et al*., 2016) and Tanzania (Bwire *et al*., 2020) due to the removal of CQ for the treatment of malaria.

The results of the present study indicated that parasites isolates with *Pfcrt* K76T mutation had higher parasitaemia than the wild-type isolates. However, we have found no significant association of this mutation with parasite density which is in agreement with the findings of Afoakwah *et al*. (2014) while other studies associated this mutation with parasite density (Al-Mekhlafi *et al*., 2011; Atrosh *et al*., 2012). On the other hand, high prevalence of this mutation was detected in the age groups 15-30 years which may be explained by more chance of this group exposed to infectious mosquito bite and CQ pressure than others. However, no significant association was found between K76T mutation and age of patients which is in agreement with findings of Atrosh *et al*. (2012) and Edogun *et al*. (2019). Similarly, no significant association of this mutation with gender was detected in this study.

In the current study, the second codon analyzed was N86Y a subset of *Pfmdr1* gene, a transporter found on the transporter membrane of the DV that facilitates the transfer of solutes including anti-malarial drugs from the cytosol to the DV (Ibraheem *et al*., 2014). All *P. falciparum* clinical isolates analyzed in the study under report carried *Pfmdr1* N86 wild-type allele. Withdrawal of CQ and use of AL for the treatment of uncomplicated falciparum resulted in the return of the wild-type genotypes for the gene under report. No single mutant *Pfmdr1* 86Y genotype was detected in this study. The current finding differs from the result of Schunk *et al*. (2006) who reported high prevalence (81%) of 86Y mutant allele two years later since the deployment of AL in Ethiopia. However, our finding is comparable with the previous reports from various parts of Ethiopia (Mekonnen *et al*., 2014; Golassa *et al*., 2015; Lo *et al*., 2017; Hailemeskel *et al*., 2019). The current high prevalence of N86 allele (CQ sensitive) showed the survival advantage of wild-type over the mutant type since the implementation of AL for the treatment of falciparum malaria in Ethiopia as evident the selection of this allele by AL elsewhere (Otienoburu *et al*., 2016).

After implementation of AL for the treatment of falciparum malaria, progressive increase in N86 allele and decrease in 86Y allele have been documented in various African countries (Asare *et al*., 2021; Chidimatembue *et al*., 2021). Selection of *Pfmdr1* N86 after implementation of AL is a common phenomenon. *In vivo* follow-up studies showed that after AL treatment significant selection of N86 allele was detected in newly infecting parasites (Sisowath *et al*., 2005; Dokomajilar *et al*., 2006). Re-infecting parasites harboring this allele (N86) and NFD haplotype have been shown to withstand LF (partner drug of ART) 35-fold and 15-fold higher compared to those harboring 86Y allele and YYY haplotype, respectively (Malmberg *et al*., 2013). Meta-analysis of thirty-one clinical trials have showed a five-fold risk of parasite recrudescence in patients infected with parasites harboring wild-type N86 allele following treatment with AL compared to patients infected with 86Y mutant parasites (Venkatesan *et al*., 2014). Similarly, allelic replacement study conducted by Veiga *et al*. (2016) revealed that substitution of N86Y mutation with N86 withstands three-fourfold higher LF and MQ concentrations compared to 86Y residues. Although as this moment there is no conclusive evidence linking N86 allele with treatment failures, high prevalence of this allele in treatment failures in Angola (Dimbu *et al*., 2021) and parasite tolerance to LF (Otienoburu *et al*., 2016; Veiga *et al*., 2016), is a clear warning signal for plausible emergence of resistance against AL.

In this study, the high prevalence of *Pfcrt* 76T allele is associated with indirect CQ drug pressure as already explained above, however; complete fixation of *Pfmdr1* N86 and absence of *Pfmdr1* 86Y allele may be the impact of AL pressure where parasites with 86Y allele are quickly cleared by AL and those with counterpart allele (N86) are being selected by the drug and spread more quickly (Venkatesan *et al*., 2014). On the other hand, in the presence of CQ pressure, parasites with *Pfcrt* 76T alleles expand more quickly than *Pfmdr1* 86Y alleles suggesting that the impact of CQ is more pronounced on *Pfcrt* gene than on *Pfmdr1* (Mehlotra *et al*., 2008).

Among 121 microscopically confirmed *P. falciparum* samples nested PCR amplification was unsuccessful for 38 samples particularly for *Pfcrt* K76T codon as was observed elsewhere (Adamu *et al*., 2020). However, 26 samples were successfully amplified for *Pfmsp1* gene. This indicated that such parasites may have low concentrated intact DNA that could not be amplified. Still, those 12 samples which were not again amplified for *Pfmsp1* indicated that

they may be microscopically false positive for *P. falciparum* (Jaleta *et al*., 2020).

5.2. Infectiousness of Symptomatic Malaria Patients to *Anopheles arabiensis* Mosquitoes

In this study 68.3% of symptomatic patients were found infectious to mosquitoes. This proportion is comparatively higher than some of the studies reported from Burkina Faso (32.6%, Ouedraogo *et al*., 2016), Ethiopia (27.2%, Tadesse *et al*., 2018), Papua New Guinea (20.9%, Timinao *et al*., 2021) and The Gambia (55.9%, Ahmad *et al*., 2021) but lower than findings from Mali (85.7%) and Cameroon (100%) (Bousema *et al*., 2012). The differences in infectiousness of the symptomatic patients may be due to gametocyte density (Bradeley *et al*., 2018), gametocyte sex ratio (Robert *et al*., 2003; Bradeley *et al.,* 2018), immune response (Bousema and Drakeley, 2011) and susceptibility of the mosquitoes (Ndo *et al*., 2016; Santos *et al*., 2022).

The study revealed that infectiousness of *P. vivax* infected patients to mosquitoes was higher as compared to *P. falciparum* infected patients. The current finding is in agreement with the previous studies that reported higher infectiousness *P. vivax* infected patients than those infected with *P. falciparum* (Tadesse *et al*., 2018; Timinao *et al*., 2021). The plausible explanations may include: (i) higher gametocyte densities in *P. vivax* infected patients compared to those infected *P. falciparum* in the current study, (ii) inherent difference in the biology of both parasites where gametocytes of *P. vivax* appear earlier in the peripheral blood (within 2-3 days after the appearance of asexual parasites) (Bantuchai *et al*., 2022) compared to those of *P. falciparum* where matured gametocytes of *P. falciparum* appear 10-12 days after the appearance of asexual stages and also take further 2-3 days to mature before they can infect mosquitoes (Bousema & Drakeley, 2011; Nilsson *et al*., 2015), (iii) *P. vivax* gametocytes are efficient in transmission at lower gametocyte densities compared to those of *P. falciparum* (Bousema *et al*., 2011; Almeida *et al*., 2021).

The results of the present study showed that 84.8% of microscopically detectable gametocyte carriers were found to infect $\geq$1 *An. arabiensis* mosquitoes, while 23.5% cases with undetectable gametocytes were capable of infecting mosquitoes The present finding is in agreement with previous findings of the higher likelihood infectivity of microscopically detectable gametocyte carriers (Tadesse *et al*., 2018). Although we did not determine gametocyte densities by molecular methods, the contribution of submicroscopic gametocyte

carriers to onward malaria transmission is evident elsewhere suggesting the presence of at least one male and one female gametocytes but undetected by microscopy diagnosis because of its sensitivity limitation (Gaye *et al*., 2015; Vallejo *et al*., 2016; Ouedraogo *et al*., 2017).

When level of infectiousness was compared among demographic characteristics, females and rural residents remained the major reservoir of *Plasmodium* parasites. Proportionally, females and rural residents were significantly more infectious to mosquitoes compared to males and urban residents, respectively. This might be because of higher gametocyte densities among females and rural residents than males and urban residents, respectively. In addition, most of the females in the current study were infected with *P. vivax* which is more infective to mosquitoes than *P. falciparum* due to the reasons already explained above. The reason for higher gametocyte densities among these groups may require further investigation. Life style may also cause this differential infectiousness of rural and urban residents. Study conducted by Coalson *et al*. (2016) showed that individuals who live in poor houses displayed higher gametocyte densities. In the current study no significant difference in infectiousness was determined among age groups although gametocytes density is the highest in age category of 25-44 compared to those age groups of $\leq$24 and $\geq$45 suggesting that in such low malaria endemic area mosquito infections occurred from all age groups without age dependency (Goncalves *et al*., 2017). This finding is in contrast to the previous studies of Churcher *et al*. (2013) and Ahmad *et al*. (2021) where mosquito infectiousness is higher in children compared to adults suggesting protective immunity increased with age (Goncalves *et al*., 2017).

5.3. Prevalence of Infected and Infectious Mosquitoes

In the current study, mosquito infection rate (21.6%) was higher than previous studies of Gaye *et al*. (2015), Tadesse *et al*. (2018) and Ahmad *et al*. (2021) who reported 1.8%, 9.1% and 15.4% mosquito infection rates, respectively. In the present study parasitaemia and gametocytaemia were good predictors of mosquito infectivity. Both parasitaemia and gametocytaemia densities positively correlated with oocyst densities. Generally, as the number of both asexual and sexual stages increased rate of mosquito infection also increased. Our findings were in agreement with the studies of Nilsson *et al*. (2015), Kiattibutr *et al*. (2017), Bradley *et al*. (2018) and Timinao *et al*. (2021) who found positive correlation between mosquito infection rates and parasite and/or gametocyte densities but contradictory to the

findings of Nina *et al*. (2017) and Santos *et al*. (2022).

Proportions of mosquito infection were also positively correlated with oocyst densities. Similar findings were demonstrated by the study of Timinao and colleagues (2021) that showed moderate and significant positive correlation between mosquito infection rates and oocyst densities for both *P. falciparum* and *P. vivax*. Compared to *P. falciparum* oocyst infected mosquitoes, higher densities of oocyst per individual mosquito and per DMFA were recorded from *P. vivax* infected participants. This difference might be due to high mean oocyst that increased with parasitaemia and gametocytaemia which is in line with previous studies (Churcher *et al*., 2013; Nina *et al*., 2017; Bradeley *et al*., 2018).

The presence or level of oocysts in the mid-gut of mosquitoes is an indicative of mosquito infectivity. In low density infections, the relationship between oocyst level and mosquito infectivity was demonstrated to be consistent, despite a subset of oocysts unable to release viable sporozoites (Stone *et al*., 2013). Although small number of mosquitoes per assay was dissected for salivary glands, nearly 61.0% of the randomly selected DMFAs with oocyst-positive mosquitoes had sporozoite infected mosquitoes. Mosquitoes with sporozoite infected salivary glands play a great role in the onward transmission of malaria to humans.

Previous study of Solarte *et al*. (2011) which is in line with our current study indicated that oocyst infection rates was significantly correlated with the percentages of sporozoite infection. This indicates that it is possible to estimate/predict infectiousness mosquito from oocyst infection alone without undergoing salivary gland dissection for sporozoite detection which require further one week post-mid-gut dissection. The current findings might help for the development and evaluation of TRIs (drugs) and/or transmissions blocking vaccines. Since the laboratories which evaluate the efficacy of such drugs and/or vaccines are located in malaria non-endemic countries. Thus, the current finding might help these laboratories to get sporozoite stages from malaria-endemic settings where such laboratories are unavailable.

In the study under report, strong correlation was found between sporozoite infection and gametocytaemia, which is in contrast with a previous study in India (Nina *et al*., 2017). Significant and positive correlation was found between oocyst and sprozoite loads which is in agreement with study elsewhere (Solarte *et al*., 2011) although negative correlation was reported when oocyst load was greater than 79 per mosquito (Nina *et al*., 2017).

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

Monitoring anti-malarial drug resistance markers and identifying individuals who contribute most to human malaria infectious reservoirs by membrane feeding assays are among the efforts of malaria control and elimination strategies. This study points out prevalence of *Pfcrt* K76T and *Pfmdr1* N86Y codons among *P. falciparum* isolates; as well as infectiousness of symptomatic malaria patients and their contribution to infectiousness of mosquitoes.

1. In the first study, the study gives an update on the prevalence of K76T and N86Y alleles at *Pfcrt* and *Pfmdr1* genes, respectively in East Shewa zone, Oromia Regional State, Ethiopia. Significantly, high prevalence (91.6%) of *Pfcrt* 76T mutation (CQ-resistant) and fixation of *Pfmdr1* N86 wild-type were detected among *P. falciparum* clinical isolates. *Pfcrt* K76 wild-type allele was recovered in non-significant proportions while no *Pfmdr1* 86Y mutation was detected in all the samples analyzed. The current high prevalence of *Pfcrt* 76T mutation is suggestive of CQ pressure, despite the official withdrawal of CQ for the treatment of *P. falciparum* for more than two decades. Similarly, fixation of *Pfmdr1* N86 allele indicates its being selected by AL in the study areas. Evidences also indicated that in the presence of CQ pressure, *Pfcrt* 76T alleles expand more quickly and reach higher frequencies than *Pfmdr1* 86Y alleles, suggesting that *Pfcrt* is under strong selection by CQ pressure compared to *Pfmdr1*gene.

2. In the second study, the findings of 63 DMFAs on colonies of *An. arabiensis* mosquitoes were described. High infectiousness of symptomatic patients was detected by DMFA; where the infectiousness was affected by both gametocyte and parasite densities. The proportion of infectious symptomatic patients was 68.3% by mosquito mid-gut dissection. Symptomatic *P. vivax* infections were more likely to infect mosquitoes compared to *P. falciparum* symptomatic infections. This differential mosquito infectivity may be due to the difference in the time required for the gametocytes maturation in *P. falciparum* and *P. vivax*. In the present study mosquito infection rate was 21.6% and parasitaemia and gametocyteamia were positively correlated with proportion of infected mosquitoes. Salivary glands examinations of mosquitoes indicated that more than half (60.7%) of the 28 randomly selected assays with oocyst positive mosquitoes resulted in infectiousness of mosquitoes.

6.2. Recommendations

1. Conclusion of the current study was restricted to relatively small sample size collected from the study areas, thus, further studies using larger sample size on the prevalence of anti-malarial drug resistance markers and genetic diversity of *P. falciparum* population is needed.

2. In this study *P. falciparum* parasites with *Pfcrt* K76T mutation are highly prevalent in the study areas, despite withdrawal of CQ treatment for uncomplicated *P. falciparum* since 1998. This might attributed to indirect but sustained CQ pressure, which is still in use for the treatment of *P. vivax*. Based on this finding we recommend careful monitoring usage of CQ for the treatment of vivax malaria.

3. Prompt and accurate malaria diagnosis is an effective means of malaria case management, in the way that it reduces malaria transmission and unnecessarily uses of anti-malarial drugs and thus reduces the emergence and spread of anti-malarial drug resistance. Co-existence of *P. falciparum* and *P. vivax* causes for the occurrence of mixed infections of the two *Plasmodium* species. Misdiagnosing mixed infections as *P. vivax* and treating with CQ was commonly encountered during the study period; this might partly contribute for persistent high circulations of *P. falciparum* parasites with *Pfcrt* K76T mutations in the study areas. Therefore, in this regard we recommend continuous trainings and performance evaluations for laboratory technologists that improve their skills to accurately diagnose malaria infections.

4. During early implementation of AL, there was high prevalence of *Pfmdr1* 86Y (mutant-type) allele in Ethiopia. In the current study fixation of *Pfmdr1* N86 (wild-type) allele indicated that AL is selecting this wild-type allele. This allele has been linked to increased resistance to LF in *in vitro* study and high prevalence of this allele was found in treatment failures in some countries. Thus, the current high prevalence of this allele could be an early warning signal for plausible emergence of resistance against AL particularly LF. Currently, AL is the only ACT based treatment against *P. falciparum* in Ethiopia, therefore; continuous monitoring efficacy should take place inorder to prevant AL failures. Also, other ACTs such as DHA-PPQ which counteract with AL should be evaluated and used as an alternative regimen for the treatment of both *P. falciparum* and *P. vivax*.

5. The current DMFA conducted to determine infectivity of symptomatic malaria infected patients to colonies of *An. arabiensis* may serve as a baseline evidence for those scholars who want to conduct further study in areas with similar epidemiological settings. Identifying individuals who contribute the most to malaria infectious reservoir will help to implement optimum malaria controlling and elimination strategies. Thus, further studies on the human and mosquito determinant factors with large sample sizes and molecular techniques are needed to guide malaria control and elimination efforts.
6. The current study includes only infectivity of symptomatic patients, thus it does not represent the whole transmission picture of the study areas. Therefore, future study should also address asymptomatic infections using microscopy and other techniques such as qPCR and circumsporozoite protein- enzyme linked immunosorbant assays.

REFERENCES

Abamecha, A., Yilma, D., Addisu, W., El-Abid, H., Ibenthal, A., Noedl, H., Yewhalaw, D., Moumni, M., & Abdissa, A. (2020). Therapeutic efficacy of artemether-lumefantrine in the treatment of uncomplicated *Plasmodium falciparum* malaria in Chewaka District, Ethiopia. *Malaria Journal*, *19*(240). https://doi.org/10.1186/s12936-020-03307-4

Abdul-Ghani, R., Al-Maktari, M.T., Al-Shibani, L.A., & Allam, A.F. (2014). A better resolution for integrating methods for monitoring *Plasmodium falciparum* resistance to antimalarial drugs. *Acta Tropica*, *137*, 44-57. 10.1016/j.actatropica.2014.04.031

Abduselam, N., Zeynudin, A., Berens-riha, N., Seyoum, D., Pritsch, M., Tibebu, H., Eba, K., Hoelscher, M., Wieser, A., & Yewhalaw, D. (2016). Similar trends of susceptibility in *Anopheles arabiensis* and *Anopheles pharoensis* to *Plasmodium vivax* infection in Ethiopia. *Parasites & Vectors*, *9*(552). https://doi.org/10.1186/s13071-016-1839-0

Adam, R., Mukhtar, M.M., Abubakar, U.F., Damudi, H.A., Muhammad, A., & Ibrahim, S.S. (2021). Polymorphism analysis *Pfmdr1* and *Pfcrt* from *Plasmodium falciparum* isolates in Northwestern Nigeria revealed the major markers associated with antimalarial resistance. *Diseases*, *9*(6). https://doi.org/10.3390/diseases 9010006

Adamu, A., Jada, M.S., Haruna, H.M.S., Yakubu, B.O., Ibrahim, M.A., Balogun, E.O., Sakura, T., Inaoka, D.K., Kita, K., Hirayama, K., Culleton, R., & Shuaibu, M.N. (2020). *Plasmodium falciparum* multidrug resistance gene-1 polymorphisms in Northern Nigeria: Implications for the continued use of artemether-lumefantrine in the region. *Malaria Journal*, *19*(439). https://doi.org/10.1186/s12936-020-03506-z

Adugna, F., Wale M., & Nibret E. (2021). Review of *Anopheles* mosquito species, abundance, and distribution in Ethiopia. *Journal of Tropical Medicine*, *2021*. https://doi.org/10.1155/2021/6726622

Ahmad, A., Soumare, H.M., Camara, M.M., Jadama, L., Gaye, P.M., Bittaye, H., Bradley, J., Achan, J., Bousema, T., D'Alessandro, U., Drakeley, C., & Moreno, M. (2021). Infectivity of patent *Plasmodium falciparum* gametocyte carriers to mosquitoes: Establishing capacity to investigate the infectious reservoir of malaria in a low-

transmission setting in The Gambia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *115*, 1462-1467. https://doi.org/10.1093/trstmh/trab087

Al-Mekhlafi, A.M., Mahdy, M.A.K., Al-Mekhlafi, H.M., Azazy, A.A., & Fong, M.Y. (2011). High frequency of *Plasmodium falciparum* chloroquine resistance marker (*Pfcrt* T76 mutation) in Yemen: An urgent need to re-examine malaria drug policy. *Parasites and Vectors*, *4*(94). https://doi.org/10.1186/1756-3305-4-94

Alelign, A., & Dejene, T. (2016). Current status of malaria in Ethiopia: evaluation of the burden, factors for transmission and prevention methods. *Acta Parasitologica Globalis*, *7*(1). https://doi.org/10.5829/idosi.apg.2016.7.1.10273

Alemayehu, G.S., Blackburn, K., Lopez, K., Cambel Dieng, C., Lo, E., Janies, D., & Golassa, L. (2021). Detection of high prevalence of *Plasmodium falciparum* histidine-rich protein 2/3 gene deletions in Assosa zone, Ethiopia: Implication for malaria diagnosis. *Malaria Journal*, *20*, 109, 1-11. https://doi.org/10.1186/s12936-021-03629-x

Alemu, A., Fuehrer, H., Getnet, G., Tessema, B., & Noedl, H. (2013). *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* in Northwest Ethiopia. *Malaria Journal*, *12*(346) https://doi.org/10.1186/1475-2875-12-346

Almeida, G.G., Costa, P.A.C., Araujo, M. da S., Gomes, G.R., Carvalho, A.F., Figueiredo, M. M., Pereira, D.B., Tada, M.S., Medeiros, J.F., Soares, I. da S., Carvalho, L.H., Kano, F. S., de Castro, M.C., Vinetz, J.M., Golenbock, D.T., Do Valle Antonelli, L.R., & Gazzinelli, R.T. (2021). Asymptomatic *Plasmodium vivax* malaria in the brazilian amazon: Submicroscopic parasitemic blood infects *Nyssorhynchus darlingi*. *PLoS Neglected Tropical Diseases*, *15*(10). https://doi.org/10.1371/journal.pntd.0009077

Andrade, C.M., Fleckenstein, H., Thomson-luque, R., Doumbo, S., Lima, N.F., Anderson, C., Hibbert, J., Hopp, C.S., Tran, T.M., Li, S., Niangaly, M., Cisse, H., Doumtabe, D., Skinner, J., Sturdevant, D., Ricklefs, S., Virtaneva, K., Asghar, M., Homann, M.V., …Crompton, P.D. (2020). Increased circulation time of *Plasmodium falciparum* underlies persistent asymptomatic infection in the dry season. *Nature Medicine*, *26*, 1929-1940. https://doi.org/10.1038/s41591-020-1084-0

Antony, H. A., Das, S., Parija, S. C., & Padhi, S. (2016). Genomics data sequence analysis of *Pfcrt* and *Pfmdr1* genes and its association with chloroquine resistance in Southeast Indian *Plasmodium falciparum* isolates. *Genomics Data*, *8*, 85-90. https://doi.org/10.1016/j.gdata.2016.04.010

Apinjoh, T.O., Ouattara, A., Titanji, V.P.K., Djimde, A., & Ngwa, A.A. (2019). Genetic diversity and drug resistance surveillance of *Plasmodium falciparum* for malaria elimination : Is there an ideal tool for resource-limited sub-Saharan Africa ? *Malaria Journal*, *18*(217), 1-12, https://doi.org/10.1186/s12936-019-2844-5

Ariey F., Witkowski B., Amaratunga C., Beghain J., Langlois A.C., Khim N., Kim, S., Duru, V., Bouchier, Ch., Ma, L., Lim, Ph., Leang, R., Duong, S., Sreng, S., Suon, S., Chuor, Ch.M., Bout7, D.M., Ménard, S., Rogers, W.O.,…Menard D. (2014). A molecular marker of artemisinin-resistant *P. falciparum* malaria. *Nature*, *505*, 50-55. https:// doi.org/10.1038/nature12876

Asale, A., Duchateau, L., Devleesschauwer, B., Huisman, G., & Yewhalaw, D. (2017). Zooprophylaxis as a control strategy for malaria caused by the vector *Anopheles arabiensis* (Diptera : Culicidae): A systematic review. *Infectious Diseases of Poverty*, *6*(160). https://doi.org/10.1186/s40249-017-0366-3

Asare, K.K., Africa, J., Mbata, J., & Opoku, Y.K. (2021). The emergence of chloroquine-sensitive *Plasmodium falciparum* is influenced by selected communities in some parts of the Central Region of Ghana. *Malaria Journal*, *20*(447). https://doi.org/10.1186/s12936-021-03985-8

Atroosh, W.M., Al-mekhlafi, H.M., Mahdy, M.A.K., & Surin, J. (2012). The detection of *Pfcrt* and *Pfmdr1* point mutations as molecular markers of chloroquine drug resistance , Pahang, Malaysia. *Malaria Journal*, *11*(251). https://doi.org/10.1186/1475-2875-11-251

Atroosh, W.M., Al-Mekhlafi, H.M., Al-Jasari, A., Sady, H., Dawaki, S.S., Elyana, F.N., Al-Areeqi, M.A., Nasr, N.A., Abdulsalam, A.M., Subramaniam, L.R., Azzani, M., Ithoi, I., Lau, Y.L., & Surin, J. (2016). Different patterns of *Pfcrt* and *Pfmdr1* polymorphism in *Plasmodium falciparum* isolates from Tehama region, Yemen. *Peer Journal*, *4*.

https://doi.org/10.7717/peerj.2191

Autino, B., Noris, A., Russo, R., & Castelli, F. (2012). Epidemiology of malaria in endemic areas. *Mediterranean Journal of Hematology and Infectious Diseases*, *4*(1). https://doi.org/10.4084/MJHID.2012.060

Awolola, T. S., Adeogun, A., Olakiigbe, A. K., Oyeniyi, T., Olukosi, Y. A., Okoh, H., Arowolo, T., Akila, J., Oduola, A., & Amajoh, C. N. (2018). Pyrethroids resistance intensity and resistance mechanisms in *Anopheles gambiae* from malaria vector surveillance sites in Nigeria. *PLOS ONE*, *13*(12).10.1371/journal.pone.0205230

Balikagala, B., Sakurai-Yatsushiro, M., Tachibana, S. I., Ikeda, M., Yamauchi, M., Katuro, O. T., Ntege, E. H., Sekihara, M., Fukuda, N., Takahashi, N., Yatsushiro, S., Mori, T., Hirai, M., Opio, W., Obwoya, P. S., Anywar, D. A., Auma, M. A., Palacpac, N. M. Q., Tsuboi, T., … Mita, T. (2020). Recovery and stable persistence of chloroquine sensitivity in *Plasmodium falciparum* parasites after its discontinued use in Northern Uganda. *Malaria Journal*, *19*(76). https://doi.org/10.1186/s12936-020-03157-0

Balkew, M., Mumba, P., Dengela, D., Yohannes, G., Getachew, D., Yared, S., Chibsa, S., Murphy, M., George, K., Lopez, K., Janies, D., Choi, S. H., Spear, J., Irish, S. R., & Carter, T. E. (2020). Geographical distribution of *Anopheles stephensi* in Eastern Ethiopia. *Parasites and Vectors*, *13*(35). https://doi.org/10.1186/s13071-020-3904-y

Bantuchai, S., Imad, H., & Nguitragool, W. (2022). *Plasmodium vivax* gametocytes and transmission. *Parasitology International*, *87*(102497). DOI:10.1016/j.parint.2021.102497

Barry, A., Bradley, J., Stone, W., Guelbeogo, M.W., Lanke, K., Ouedraogo, A., Soulama, I., Nébié, I., Serme, S.S., Grignard, L., Patterson, C., Wu, L., Briggs, J.J., Janson, O., Awandu, S.S., Ouedraogo, M., Tarama, C.W., Kargougou, D., Zongo, S.,… Bousema, T. (2021). Higher gametocyte production and mosquito infectivity in chronic compared to incident *Plasmodium falciparum* infections. *Nature Communications*, *12*(2443). https://doi.org/10.1038/s41467-021-22573-7

Basu, S., & Sahi, P. K. (2017). Malaria : An Update. *Indian Journal of Pediatrics*, *84*(7), 521–528. https://doi.org/10.1007/s12098-017-2332-2

Beavogui, A.H., DIawara, E.Y., Cherif, M.S., Delamou, A., DIallo, N., Traore, A., Millimouno, P., Camara, D., Sylla, M.M., Toure, A.A., DIallo, M.S., Toure, S., Togo, A., Camara, G., Kourouma, K., Sagara, I., DIcko, A., & Djimde, A. (2021). Selection of *Pfcrt* 76T and *Pfmdr1* 86Y mutant *Plasmodium falciparum* after treatment of uncomplicated malaria with artesunate-amodiaquine in Republic of Guinea. *Journal of Parasitology*, *107*(5), 778-782, https://doi.org/10.1645/19-199

Belete, T. (2020). Recent progress in the development of new antimalarial drugs with novel targets. *Drug Design, Development and Therapy*, *14*, 3875-3889. https://doi.org/10.2147/DDDT.S265602

Benelli, G., & Beier, J.C. (2017). Current vector control challenges in the fight against malaria. *Acta Tropica*, *174*, 91-96. https://doi.org/10.1016/j.actatropica.2017.06.028

Berhane, A., Anderson, K., Mihreteab, S., Gresty, K., Rogier, E., Mohamed, S., Hagos, F., Embaye, G., Chinorumba, A., Zehaie, A., Dowd, S., Waters, N.C., Gatton, M.L., Udhayakumar, V., Cheng, Q., & Cunningham, J. (2018). Major threat to malaria control programs by *Plasmodium falciparum* lacking histidine-rich protein-2, Eritrea. *Emerging Infectious Diseases*, *24*(3), 462-470. https://doi.org/10.3201/eid2403.171723

Bharti, P.K., Shukla, M.M., Sharma Y.D. & Singh, N. (2012). Genetic diversity in the block 2 region of the merozoite surface protein-1 of *Plasmodium falciparum* in central India. *Malaria Journal*, *11*(78), http://www.malariajournal.com/content/11/1/78

Bhatt, S., Weiss, D.J., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., Battle, K.E., Moyes, C.L., Henry, A., Eckhoff, P.A., Wenger, E.A., Briët, O., Penny, M.A., Smith, T. A., Bennett, A., Yukich, J., Eisele, T.P., Griffin, J.T., Fergus, C.A.,…Gething, P.W. (2015). The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature*, *526*, 207-211. https://doi.org/10.1038/nature15535

Bian, G., Joshi, D., Dong, Y., Lu, P., Zhou, G., Pan, X., Xu, Y., Dimopoulos, G., & Xi, Z. (2013). Wolbachia invades *Anopheles stephensi* populations and induces refractoriness to

Plasmodium infection. *Science*, *340*, 748-751. https://doi.org/10.1126/science.1236192

Bousema, T., & Drakeley, C. (2011). Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. *Clinical Microbiology Reviews*, *24*(2), 377-410. https://doi.org/10.1128/CMR.00051-10

Bousema, T., Sutherland, C.J., Churcher, T.S., Mulder, B., Gouagna, L.C., Riley, E.M., Targett, G.A.T., & Drakeley, C.J. (2011). Human immune responses that reduce the transmission of *Plasmodium falciparum* in African populations. *International Journal for Parasitology*, *41*(3-4), 293-300. https://doi.org/10.1016/j.ijpara.2010.09.008

Bousema, T., Dinglasan, R.R., Morlais, I., Gouagna, L.C., van Warmerdam, T., Awono-Ambene, P.H., Bonnet, S., Diallo, M., Coulibaly, M., Tchuinkam, T., Mulder, B., Targett, G., Drakeley, C., Sutherland, C., Robert, V., Doumbo, O., Touré, Y., Graves, P.M., Roeffen, W.,…Churcher, T.S. (2012). Mosquito feeding assays to determine the infectiousness of naturally infected *Plasmodium falciparum* gametocyte carriers. *PLoS ONE*, *7*(8). https://doi.org/10.1371/journal.pone.0042821

Bousema, T., Churcher, T.S., Morlais, I., & Dinglasan, R.R. (2013). Can field-based mosquito feeding assays be used for evaluating transmission-blocking interventions? *Trends in Parasitology*, *29*(2), 53-59. https://doi.org/10.1016/j.pt.2012.11.004

Bousema, T., & Drakeley, C. (2017). Determinants of malaria transmission at the population level. *Cold Spring Harbor Perspective Medicine*. *7*(12). 10.1101/cshperspect.a025510

Bradley, J., Stone, W., Da, D.F., Morlais, I., Dicko, A., Cohuet, A., Guelbeogo, W.M., Mahamar, A., Nsango, S., Soumaré, H.M., Diawara, H., Lanke, K., Graumans, W., Siebelink-Stoter, R., van de Vegte-Bolmer, M., Chen, I., Tiono, A., Gonçalves, B.P., Gosling, R.,…Bousema, T. (2018). Predicting the likelihood and intensity of mosquito infection from sex specific *Plasmodium falciparum* gametocyte density. *ELife*, *7*. https://doi.org/10.7554/eLife.34463.

Bray, P.G., Martin, R.E., Tilley, L., Ward, S.A., Kirk, K., & Fidock, D.A. (2005). Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. *Molecular Microbiology*, *56*(2), 323-333. https://doi.org/10.1111/j.1365-2958.2005.04556.x

Bray, P.G., Mungthin, M., Hastings, I.M., Biagini, G.A., Saidu, D.K., Lakshmanan, V., Johnson, D.J., Hughes, R.H., Stocks, P.A., O'Neill, P.M., Fidock, D.A., Warhurst, D. C., & Ward, S. A. (2006). PfCRT and the trans-vacuolar proton electrochemical gradient: regulating the access of chloroquine to ferriprotoporphyrin IX. *Molecular Microbiology*, *62*(1), 238-251. https://doi.org/10.1111/j.1365-2958.2006.05368.x

Bugssa, G., & Tedla, K. (2020). Feasibility of malaria elimination in Ethiopia. *Ethiopian Journal of Health Sciences, 30*(4), 607-614. https://doi.org/10.4314/ejhs.v30i4.16

Bushman, M., Antia, R., Udhayakumar, V., & de Roode, J. C. (2018). Within-host competition can delay evolution of drug resistance in malaria. *PLoS Biology*, *16*(8). https://doi.org/10.1371/journal.pbio.2005712.

Bwire, G.M., Ngasala, B., Mikomangwa, W.P., Kilonzi, M., & Kamuhabwa, A.A.R. (2020). Detection of mutations associated with artemisinin resistance at K13- propeller gene and a near complete return of chloroquine susceptible falciparum malaria in Southeast of Tanzania. *Scientific Reports, 10*(3500). https://doi.org/10.1038/s41598-020-60549-7.

Camarda, G., Jirawatcharadech, P., Priestley, R.S., Saif, A., March, S., Wong, M.H.L., Leung, S., Miller, A.B., Baker, D.A., Alano, P., Paine, M.J.I., Bhatia, S.N., O'Neill, P.M., Ward, S.A., & Biagini, G.A. (2019). Antimalarial activity of primaquine operates via a two-step biochemical relay. *Nature Communications*, *10*(3226). https://doi.org/10.1038/s41467-019-11239-0

Carter, T.E., Yared, S., Gebresilassie, A., Bonnell, V., Damodaran, L., Lopez, K., Ibrahim, M., Mohammed, S. & Janies, D. (2018). First detection of *Anopheles stephensi* Liston , 1901 (Diptera : culicidae) in Ethiopia using molecular and morphological approaches. *Acta Tropica*, *188*, 180-186. https://doi.org/10.1016/j.actatropica.2018.09.001

Chahar, M., Mishra, N., Anvikar, A., Dixit, R., & Valecha, N. (2017). Establishment and application of a novel isothermal amplification assay for rapid detection of chloroquine resistance (K76T) in *Plasmodium falciparum*. *Scientific Reports, 7*(41119). https://doi.org/10.1038/srep41119.

Challenger, J.D., Mesa, D.O., Da, D.F., Lefèvre, T., Cohuet, A., Churcher, T.S., & Yerbanga, R S. (2021). Predicting the public health impact of a malaria transmission-blocking vaccine. *Nature Communications*, *12*(1494). https://doi.org/10.1038/s41467-021-21775-3

Chidimatembue, A., Svigel, S.S., Mayor, A., Aíde, P., Nhama, A., Nhamussua, L., Nhacolo, A., Bassat, Q., Salvador, C., Enosse, S., Saifodine, A., Carvalho, E. De, Candrinho, B., Zulliger, R., Goldman, I., & Udhayakumar, V. (2021). Molecular surveillance for polymorphisms associated with artemisinin-based combination therapy resistance in *Plasmodium falciparum* isolates collected in Mozambique, 2018. *Malaria Journal*, *20*(398). https://doi.org/10.1186/s12936-021-03930-9

Churcher, T.S., Bousema, T., Walker, M., Drakeley, C., Schneider, P., Ouédraogo, A.L., & Basáñez, M.G. (2013). Predicting mosquito infection from *Plasmodium falciparum* gametocyte density and estimating the reservoir of infection. *ELife*, *2*, https://doi.org/10.7554/eLife.00626.

Coalson, J.E., Walldorf, J.A., Cohee, L.M., Ismail, M.D., Mathanga, D., Cordy, R.J., Marti, M., Taylor, T.E., Seydel, K.B., Laufer, M.K., & Wilson, M.L. (2016). High prevalence of *Plasmodium falciparum* gametocyte infections in school-age children using molecular detection: Patterns and predictors of risk from a cross-sectional study in Southern Malawi. *Malaria Journal*, *15*(527). https://doi.org/10.1186/s12936-016-1587-9

Conrad, M.D., LeClair, N., Arinaitwe, E., Wanzira, H., Kakuru, A., Bigira, V., Muhindo, M., Kamya, M.R., Tappero, J.W., Greenhouse, B., Dorsey, G., & Rosenthal, P.J. (2014). Comparative impacts over 5 years of artemisinin-based combination therapies on *Plasmodium falciparum* polymorphisms that modulate drug sensitivity in Ugandan children. *Journal of Infectious Diseases*, *210*, 344-353. https://doi.org/10.1093/infdis/jiu141

Costa, G.L., Amaral, L.C., Jesus, C., Fontes, F., & Carvalho, L.H. (2017). Assessment of copy number variation in genes related to drug resistance in *Plasmodium vivax* and *Plasmodium falciparum* isolates from the Brazilian Amazon and a systematic review of the literature. *Malaria Journal*, *16*(152). https://doi.org/10.1186/s12936-017-1806-z

Coulibaly, M.B., Gabriel, E.E., Sinaba, Y., Sylla, D., Sacko, A., Sylla, L., Coulibaly, B., Hume, J. C.C., Baber, I., Assadou, M.H., Sagara, I., Wu, Y., Healy, S.A., Doumbo, O., Traore, S.F., & Duffy, P.E. (2017). Optimizing direct membrane and direct skin feeding assays for *Plasmodium falciparum* transmission-blocking vaccine trials in Bancoumana, Mali. *American Journal of Tropical Medicine and Hygein, 97*(3), 719-725. https://doi.org/10.4269/ajtmh.16-1000

Dabaro, D., Birhanu, Z., Negash, A., Hawaria, D., & Yewhalaw, D. (2021). Effects of rainfall, temperature and topography on malaria incidence in elimination targeted district of Ethiopia. *Malaria Journal*, *20*(104). https://doi.org/10.1186/s12936-021-03641-1

Dahlström, S., Aubouy, A., Maïga-Ascofaré, O., Faucher, J. F., Wakpo, A., Ezinmègnon, S., Massougbodji, A., Houzé, P., Kendjo, E., Deloron, P., Le Bras, J., & Houzé, S. (2014). *Plasmodium falciparum* polymorphisms associated with ex vivo drug susceptibility and clinical effectiveness of artemisinin-based combination therapies in Benin. *Antimicrobial Agents and Chemotherapy*, *58*(1). https://doi.org/10.1128/AAC.01790-12

Daskum, A.M., Chessed, G., Qadeer, M.A., & Tijjani, M. (2021). Antimarial chemotherapy, mechanism of action and resistance to major antimalarial drugs in clinical use: A review. *Microbes and Infectious Diseases*, *2*(1), 130-142. https://doi.org/10.21608/mid.2020.43941.1064

Datoo, M.S., Natama, M.H., Somé, A., Traoré, O., Rouamba, T., Bellamy, D., Yameogo, P., Valia, D., Tegneri, M., Ouedraogo, F., Soma, R., Sawadogo, S., Sorgho, F., Derra, K., Rouamba, E., Orindi, B., Ramos Lopez, F., Flaxman, A., Cappuccini, F.,…Tinto, H. (2021). Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: A randomised controlled trial. *The Lancet, 397,* 1809-1818. https://doi.org/10.1016/S0140-6736(21)00943-0

Datoo, M.S., Natama, H.M., Somé, A., Bellamy, D., Traoré, O., Rouamba, T., Tahita, M.C., Ido, N.F.A., Yameogo, P., Valia, D., Millogo, A., Ouedraogo, F., Soma, R., Sawadogo, S., Sorgho, F., Derra, K., Rouamba, E., Ramos-Lopez, F., Cairns, M.,…Tinto, H. (2022). Efficacy and immunogenicity of R21/Matrix-M vaccine against clinical malaria after 2 years' follow-up in children in Burkina Faso: A phase 1/2b randomised controlled trial.

The Lancet Infectious Diseases, https://doi.org/10.1016/s1473-3099(22)00442-x.

Dijkman, P.M., Marzluf,T., Zhang, Y., Chang, S-y. S., Helm, D., Lanzer, M., Bujard, H. & Kudryashev, M. (2021). Structure of the merozoite surface protein 1 from *Plasmodium falciparum*. *Science Advance, 7(23),* https://doi: 10.1126/sciadv.abg0465

Dimbu, P.R., Horth, R., Cândido, A.L.M., Ferreira, C.M., Caquece, F., Garcia, L.E.A., André, K., Pembele G., Jandondo, D., Bondo, B.J., Andrade, B.N., Labuda, S., de León, G.P., Kelley, J., Patel, D., Svigel, S.S., Talundzic, E., Lucchi, N., Morais, J.F.M.,…& Plucin M.M. (2021). Continued low efficacy of artemether-lumefantrine in Angola in 2019. *Antimicrobial Agents & Chemotherapy*, *65*(2). https://doi.org/.org/10.1128/AAC.01949-20

Djimde, A., Pharm, D., Ogobara, K., Doumbo, M.D., Ph.D., Joseph, F., Cortese, B.S., Kayento, M.D., & Safi, M.D. (2001). A molecular marker for chloroquine resistant falciparum malaria. *The New Ingland Journal of Medicine*, *344*(4), 257-263, http://www.ncbi.nlm.nih.gov/pubmed/11172152

Dokomajilar, C., Nsobya, S.L., Greenhouse, B., Rosenthal, P.J., & Dorsey, G. (2006). Selection of *Plasmodium falciparum Pfmdr*1 alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. *Antimicrobial Agents and Chemotherapy*, *50*(5), 1893-1895. https://doi.org/10.1128/AAC.50.5.1893-1895.2006

Duffy, P.E., & Gorres, J.P. (2020). Malaria vaccines since 2000 : progress, priorities, products. *Vaccines*, *5*(48). https://doi.org/10.1038/s41541-020-0196-3

Ebel, E.R., Reis, F., Petrov, D.A., & Beleza, S. (2021). Historical trends and new surveillance of *Plasmodium falciparum* drug resistance markers in Angola. *Malaria Journal*, *20*(175). https://doi.org/10.1186/s12936-021-03713-2

Ecker, A., Lehane, A.M., Clain, J., & Fidock, D.A. (2012). PfCRT and its role in antimalarial drug resistance. *Trends in Parasitology*, *28*(11), 504-514. DOI: 10.1016/j.pt.2012.08.002

Edogun, H.A., Daramola, G.O., Ojerinde, A.O., Esan, C.O., & Adegbuyi, A.T. (2019). Prevalence of mutant alleles responsible for chloroquine resistance among *Plasmodium falciparum* isolates in North Central, Nigeria. *Journal of Advances in Biology and*

Biotechnology, *20*(3). https://doi.org/10.9734/JABB/2018/v20i330078

Elbadry, M.A., Existe, A., Victor, Y.S., Memnon, G., Fukuda, M., Dame, J.B., Yowell, C.A., & Okech, B.A. (2013). Survey of *Plasmodium falciparum* multidrug resistance-1 and chloroquine resistance transporter alleles in Haiti. *Malaria Journal*, *12*(426). https://doi.org/10.1186/1475-2875-12-426.

EPHI. (2017). *Anopheles* mosquito rearing and insectary handling guideline. Ethiopian Public Health Institute, Addis Ababa, Ethiopia

Fakruddin, M., Mazumdar, R., Chowdhury, A., & Mannan, K. (2012). Nucleic acid sequence based amplification (NASBA)-prospects and applications. *International Journal of Life Science and Pharma Research*, *2*(1), 106-121. ISSN 2250-0480

Fidock, D.A., Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., Ferdig, M.T., Ursos, L.M.B., Sidhu, A. bir S., Naudé, B., Deitsch, K.W., Su, X., Wootton, J.C., Roepe, P.D., & Wellems, T.E. (2000). Mutations in the *Plasmodium falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular Cell*, *6*(4), 861-871. https://doi.org/10.1016/s1097-2765(05)00077-8

File, T., Chekol, T., Solomon, G., Dinka, H., & Golassa, L. (2021). Detection of high frequency of MAD20 allelic variants of *Plasmodium falciparum merozoite surface protein 1* gene from Adama and its surroundings , Oromia , Ethiopia. *Malaria Journal*, *20*(385). https://doi.org/10.1186/s12936-021-03914-9

Foote, S.J., Kyle, D.E., Martin, R.K., Oduola, A., Forsyth, K., Kemp, D.J., & Cowman, A.F. (1990). Several alleles of multidrug resistance gene are closely linked to chloroquine resistance *Plasmodium falciparum*. *Nature*, *345*, 255-258.

Fontecha, G., Pinto, A., Archaga, O., Betancourth, S., Escober, L., Henríquez, J., Valdivia, H.O., Montoya, A. & Mejía, R.E. (2021). Assessment of *Plasmodium falciparum* anti-malarial drug resistance markers in *pfcrt* and *pfmdr1* genes in isolates from Honduras and Nicaragua, 2018-2021. *Malaria Journal, 20*(465). https://doi.org/10.1186/s12936-021-03977-8

Frosch, A.E.P., Laufer, M.K., & Mathanga, D.P. (2014). Return of widespread chloroquine-sensitive *Plasmodium falciparum* to Malawi. *The Journal of Infectious Diseases*, *210*, 1110-1111. https://doi.org/10.1093/infdis/jiu216

Fukuda, N., Tachibana, S.I., Ikeda, M., Sakurai-Yatsushiro, M., Balikagala, B., Katuro, O.T., Yamauchi, M., Emoto, S., Hashimoto, M., Yatsushiro, S., Sekihara, M., Mori, T., Hirai, M., Opio, W., Obwoya, P.S., Auma, M.A., Anywar, D.A., Kataoka, M., Palacpac, N.M. Q.,…Mita, T. (2021). Ex vivo susceptibility of *Plasmodium falciparum* to antimalarial drugs in Northern Uganda. *Parasitology International*, *81*(102277). https://doi.org/10.1016/j.parint.2020.102277.

Gadalla, N.B., Abdallah, T.M., Atwal, S., Sutherland, C.J., & Adam, I. (2013). Selection of *Pfdhfr*/*Pfdhps* alleles and declining artesunate/sulphadoxine- pyrimethamine efficacy against *Plasmodium falciparum* eight years after deployment in Eastern Sudan. *Malaria Journal*, *12*(255). https://doi.org/10.1186/1475-2875-12-255

Gari, T., & Lindtjørn, B. (2018). Reshaping the vector control strategy for malaria elimination in Ethiopia in the context of current evidence and new tools : opportunities and challenges. *Malaria Journal*, *17*(454). https://doi.org/10.1186/s12936-018-2607-8

Gaye, A., Bousema, T., Libasse, G., Ndiath, M.O., Konate, L., Faye, O., Sokhna, C., & Jawara, M. (2015). Infectiousness of the human population to *Anopheles arabiensis* by direct skin feeding in an area hypoendemic for malaria in Senegal. *American Journal of Tropical Medicine and Hygein*, *92*(3), 648-652. https://doi.org/10.4269/ajtmh.14-0402

Genc, A., Eroglu, F., & Koltas, I.S. (2010). Detection of *Plasmodium vivax* by nested PCR and real-time PCR. *Korean Journal of Parasitology*, *48*(2), 99-103. 10.3347/kjp.2010.48.2.99

Gil, J. P., & Krishna, S. (2017). *Pfmdr1* (*Plasmodium falciparum multidrug drug resistance 1* gene): a pivotal factor in malaria resistance to artemisinin combination therapies. *Expert Review of Anti-Infective Therapy*, *15*(6), 527-543. DOI:10.1080/14787210.2017.1313703

Golassa L., Baliraine, F.N., Enweji, N., Erko, B., Swedberg, G., & Aseffa, A. (2015). Microscopic and molecular evidence of the presence of asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* infections in an area with low, seasonal and unstable

malaria transmission in Ethiopia. *BMC Infectious Diseases*, *15*(310). 10.1186/s12879-015-1070-1.

Golassa, L., Enweji, N., Erko, B., Aseffa, A., & Swedberg, G. (2014). High prevalence of *Pfcrt*-CVIET haplotype in isolates from asymptomatic and symptomatic patients in South Central Oromia, Ethiopia. *Malaria Journal*, *13*(120). https://doi.org/10.1186/1475-2875-13-120

Golassa, L., Kamugisha, E., Ishengoma, D.S., Baraka, V., Shayo, A., Baliraine, F.N., Enweji, N., Erko, B., Aseffa, A., Choy, A., & Swedberg, G. (2015). Identification of large variation in *Pfcrt* , *Pfmdr -1* and *Pfubp -1* markers in *Plasmodium falciparum* isolates from Ethiopia and Tanzania. *Malaria Journal*, *14*(264). DOI:10.1186/s12936-015-0783-3

Golassa, L., & White, M.T. (2017). Population-level estimates of the proportion of *Plasmodium vivax* blood-stage infections attributable to relapses among febrile patients attending Adama malaria Diagnostic Center, East Shoa zone, Oromia, Ethiopia. *Malaria Journal*, *16*(307). https://doi.org/10.1186/s12936-017-1944-3.

Gonçalves, B.P., Kapulu, M.C., Sawa, P., Guelbéogo, W.M., Tiono, A.B., Grignard, L., Stone, W., Hellewell, J., Lanke, K., Bastiaens, G.J.H., Bradley, J., Nébié, I., Ngoi, J.M., Oriango, R., Mkabili, D., Nyaurah, M., Midega, J., Wirth, D.F., Marsh, K.,…Bousema, T. (2017). Examining the human infectious reservoir for *Plasmodium falciparum* malaria in areas of differing transmission intensity. *Nature Communications*, *8*(1133). https://doi.org/10.1038/s41467-017-01270-4

Goswami, D., Dhiman, S., Rabha, B., Kumar, D., Baruah, I., Veer, V., Bhola, R.K., & Sharma, D.K. (2014). High prevalence of *Pfcrt* K76T and *mdr1* N86Y mutations in Sonitpur district of Assam, India. *Journal of Parasitic Diseases*, *38*(3), 250-254. https://doi.org/10.1007/s12639-013-0298-1

Greenwood, B. (2005). Malaria vaccines: Evaluation and implementation. *Acta Tropica*, *95*(3), 298-304. https://doi.org/10.1016/j.actatropica.2005.04.017

Hailemeskel, E., Menberu, T., Shumie, G., Behaksra, S., Chali, W., Ke, M., Belachew, M., Shitaye, G., Petros, B., Bousema, T., Tadesse, F. G., & Gadisa, E. (2019). Prevalence of

Plasmodium falciparum Pfcrt and *Pfmdr1* alleles in settings with different levels of *Plasmodium vivax* co-endemicity in Ethiopia. *International Journal of Parasitology: Drugs and Drug Resistance, 11*, 8-12. https://doi.org/10.1016/j.ijpddr.2019.09.002

Han, ET. (2013). Loop-mediated isothermal amplification test for the molecular diagnosis of malaria. *Expert Review of Molecular Diagnosis*, *13*(2), 205–218. https://doi.org/10.1586/ERM.12.144

Heuchert, A., Abduselam, N., Zeynudin, A., Eshetu, T., Loscher, T., Wieser, A., Pritsch, M.. & Berens-Riha, N. (2015). Molecular markers of anti-malarial drug resistance in Southwest Ethiopia over time: Regional surveillance from 2006 to 2013. *Malaria Journal*, *14*(208). https://doi.org/10.1186/s12936-015-0723-2

Han, E. (2013). Loop-mediated isothermal amplification test for the molecular diagnosis of malaria. *Expert Review Molecular Diagnosis*, *13*(2), 205-218. ISSN 1473-7159

Hogan, A. B., Winskill, P., & Ghani, A. C. (2020). Estimated impact of RTS,S/AS01 malaria vaccine allocation strategies in sub-Saharan Africa: a modelling study. *PLoS Medicine, 17*(11). https://doi.org/10.1371/journal.pmed.1003377

Holder A.A. (2009). The carboxy-terminus of merozoite surface protein 1: structure, specific antibodies and immunity to malaria. Parasitology, 136, 1445-1456. https://doi.org/ 0.1017/S0031182009990515

Huang, B., Wang, Q., Deng, C., Wang, J., Yang, T., Huang, S., Su, X.Z., Liu, Y., Pan, L., Li, G., Li, D., Zhang, H., Bacar, A., Abdallah, K.S., Attoumane, R., Mliva, A.M.S.A., Zheng, S., Xu, Q., Lu, F.,…Song, J. (2016). Prevalence of crt and mdr-1 mutations in *Plasmodium falciparum* isolates from Grande Comore island after withdrawal of chloroquine. *Malaria Journal*, *15*(414). https://doi.org/10.1186/s12936-016-1474-4

Ibraheem, Z.O., Majid, R.A., Noor, S.M., Sedik, H.M., & Basir, R. (2014). Role of different *Pfcrt* and *Pf mdr-1* mutations in conferring resistance to antimalaria drugs in *Plasmodium falciparum*. *Malaria Research and Treatment, 2014*. DOI:10.1155/2014/950424

Ibrahim, M., Steenkeste, N., Khim, N., Adam, H., Konaté, L., Coppée, J., Ariey, F., & Duchemin, J. (2009). Field-based evidence of fast and global increase of *Plasmodium falciparum* drug-resistance by DNA-microarrays and PCR/RFLP in Niger. *Malaria Journal*, *8*(32). https://doi.org/10.1186/1475-2875-8-32

Ikegbunam, M.N., Nkonganyi, C.N., Thomas, B.N., Esimone, C.O., Velavan, T.P., & Ojurongbe, O. (2019). Analysis of *Plasmodium falciparum Pfcrt* and *Pfmdr1* genes in parasite isolates from asymptomatic individuals in Southeast Nigeria 11 years after withdrawal of chloroquine. *Malaria Journal, 18*(343). DOI:10.1186/s12936-019-2977-6

Imai, K., Tarumoto, N., Runtuwene, L.R., Sakai, J., Hayashida, K., Eshita, Y., Maeda, R., Tuda, J., Ohno, H., Murakami, T., Maesaki, S., & Suzuki, Y. (2018). An innovative diagnostic technology for the codon mutation C580Y in kelch13 of *Plasmodium falciparum* with MinION nanopore sequencer. *Malaria Journal*, *17*(217). https://doi.org/10.1186/s12936-018-2362-x

Ippolito, M.M., Pringle, J.C., Siame, M., Katowa, B., Aydemir, O., Oluoch, P.O., Huang, L., Aweeka, F.T., Bailey, J.A., Juliano, J.J., Meshnick, S.R., Shapiro, T.A., Moss, W.J., & Thuma, P.E. (2020). Therapeutic efficacy of artemether-lumefantrine for uncomplicated falciparum malaria in Northern Zambia. *American Journal of Medicine & Hygeine, 103*(6), 2224-2232. https://doi.org/10.4269/ajtmh.20-0852

Jaleta, F., Garoma, G., & Gerenfes, T. (2020). Evaluation of malaria microscopy diagnosis performance in public hospitals of Eastern and Central part of Oromia Region, Ethiopia, 2019. *Pathology and Laboratory Medicine International*, *12*. DOI:10.2147/plmi.s259159

Johora, F.T., Elahi, R., Nima, M.K., Hossain, M.S., Rashid, H., Kibria, M.G., Mohon, A.N., Khan, W.A., Haque, R., & Alam, M.S. (2021). Persistence of markers of chloroquine resistance in *Plasmodium falciparum* in Bangladesh. *American Journal of Tropical Medicine and Hygiene, 104*(1), 276-282. https://doi.org/10.4269/AJTMH.20-0415

Kamau, E., Tolbert, L.S., Kortepeter, L., Pratt, M., Nyakoe, N., Muringo, L., Ogutu, B., Waitumbi, J.N., & Ockenhouse, C. F. (2011). Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for detection and

quantitation of *Plasmodium* by amplifying RNA and DNA of the 18SrRNA genes. *Journal of Clinical Microbiology*, *49*(8), 2946-2953. https://doi.org/10.1128/JCM.00276-11

Kassaza, K., Long, A.C., Mcdaniels, J.M., Andre, M., Fredrickson, W., Nyehangane, D., Orikiriza, P., Operario, D.J., Bazira, J., Amumpaire, J.A.M., Moore, C.C., Guler, J.L., & Ii, Y.B. (2021). Surveillance of *Plasmodium falciparum Pfcrt* haplotypes in Southwestern Uganda by high- resolution melt analysis. *Malaria Journal*, *20*(114). https://doi.org/10.1186/s12936-021-03657-7

Ketema, T., Bacha, K., Getahun, K., Del Portillo, H.A., & Bassat, Q. (2021). *Plasmodium vivax* epidemiology in Ethiopia 2000-2020: A systematic review and meta-analysis. *PLoS Neglected Tropical Diseases*, *15*(9). DOI:10.1371/journal.pntd.0009781.

Khammanee, T., Sawangjaroen, N., Buncherd, H., & Tun, A.W. (2021). A LAMP-SNP assay detecting C580Y mutation in *Pfkelch13* gene from clinically dried blood spot samples. *Korean Journal of Parasitology*, *59*(1), 15-22. https://doi.org/10.3347/kjp.2021.59.1.15

Khan, A.Q., Khan, A.Q., Pernaute-Lau, L., Pernaute-Lau, L., Khattak, A.A., Luijcx, S., Aydin-Schmidt, B., Hussain, M., Khan, T.A., Mufti, F.U., & Morris, U. (2020). Surveillance of genetic markers associated with *Plasmodium falciparum* resistance to artemisinin-based combination therapy in Pakistan, 2018-2019. *Malaria Journal*, *19*(206). https://doi.org/10.1186/s12936-020-03276-8

Kiattibutra, K., Roobsoonga, W., Sriwichaib, P., Saeseua, T., Rachaphaewa, N., Suansomjitc, Ch., Buates, S., Obadia, T., Mueller, I., Cui, L., Nguitragool, W., & Sattabongkot, J. (2017). Infectivity of symptomatic and asymptomatic *Plasmodium vivax* infections to a Southeast Asian vector, *Anopheles dirus*. *International Journal of Parasitology*, *47*(2-3), 163-170. doi:10.1016/j.ijpara.2016.10.006

Killeen, G.F., Tatarsky, A., Diabate, A., Chaccour, C.J., Marshall, J.M., Okumu, F.O., Brunner, S., Newby, G., Williams, Y.A., Malone, D., Tusting, L.S., & Gosling, R.D. (2017). Developing an expanded vector control toolbox for malaria elimination. *BMJ Global Health*, *2*. https://doi.org/10.1136/bmjgh-2016-000211

Konaté, A., Gnagne, P.A., Bédia-Tanoh, V.A., Amiah-Droh, M., Tano, D.K., Ignace Eby Menan, H., & Yavo, W. (2018). Low rates of *Plasmodium falciparum Pfcrt* K76T mutation in three sentinel sites of malaria monitoring in Côte d'Ivoire. *Acta Parasitologica, 63*(4), 795-801. https://doi.org/10.1515/ap-2018-0096

Kublin, J.G., Cortese, J.F., Njunju, E.M., Mukadam, R.A.G., Wirima, J.J., Kazembe, P.N., Djimdé, A.A., Kouriba, B., Taylor, T.E., & Plowe, C.V. (2003). Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *Journal of Infectious Diseases, 187*, 1870-1875. https://doi.org/10.1086/375419

Kumar, Y., Bansal, S., & Jaiswal, P. (2017). Loop-mediated isothermal amplification (LAMP): A rapid and sensitive tool for quality assessment of meat products. *Comprehensive Reviews in Food Science and Food Safety*, *16*, 1359-1378. https://doi.org/10.1111/1541-4337.12309

Kumar, S., Bhardwaj, T.R., Prasad, D.N., & Singh, R.K. (2018). Drug targets for resistant malaria: historic to future perspectives. *Biomedicine and Pharmacotherapy*, *104*, 8-27. https://doi.org/10.1016/j.biopha.2018.05.009

Laufer, M.K., & Plowe, C.V. (2004). Withdrawing antimalarial drugs: impact on parasite resistance and implications for malaria treatment policies. *Drug Resistance Updates*, *7*, 279-288. https://doi.org/10.1016/j.drup.2004.08.003

Laurens, M.B. (2020). RTS,S/AS01 vaccine (Mosquirix^{TM}): An overview. *Human Vaccines and Immunotherapeutics*, *16*(3), 480-489.DOI: 10.1080/21645515.2019.1669415

Lehane, A.M., & Kirk, K. (2008). Chloroquine resistance-conferring mutations in *Pfcrt* give rise to a chloroquine-associated H + leak from the malaria parasite's digestive vacuole. *Antimicrobial Agents & Chemotherapy*, *52*(12), 4374–4380. DOI:10.1128/AAC.00666-08

Lin, C.S., Uboldi, A.D., Epp, Ch., Bujard, H., Tsuboi, T., Peter E. Czabotar, P.E. & Cowman, A.F. (2016). Multiple *Plasmodium falciparum* merozoite surface protein 1 complexes mediate merozoite binding to human erythrocytes. T*he Journal of Biological Chemistry*, *291*(14), 7703-7715. https://doi.org/10.1074/jbc.M115.698282

Lin, J.T., Ubalee, R., Lon,C., Balasubramanian, S., Kuntawunginn, W., Rahman, R., Saingam, P., & Heng, T.K. (2016). Microscopic *Plasmodium falciparum* gametocytemia and infectivity to mosquitoes in Cambodia. *The Journal of Infectious Diseases*, *213*, 1491-1494. https://doi.org/10.1093/infdis/jiv599

Lo, E., Hemming-schroeder, E., Yewhalaw, D., Nguyen, J., Kebede, E., Zemene, E., Getachew, S., Tushune, K., Zhong, D., Zhou, G., Petros, B., & Yan, G. (2017). Transmission dynamics of co-endemic *Plasmodium vivax* and *Plasmodium falciparum* in Ethiopia and prevalence of antimalarial resistant genotypes. *PLoS Neglected Tropical* Diseases, *11*(7). https://doi.org/10.1371/journal.pntd.0005806

Lopez, A.R., Ghansah, A., Nyamordey, Ch. & Brown, Ch (2022). Comparison of clinical, microscopic and rapid diagnostic test methods in the diagnosis of *Plasmodium falciparum* malaria in the four districts in the Eastern Region, Ghana. *Health Sciences Investigations Journal*, *3*(1), 320-326. https://doi.org/10.46829/hsijournal.2022.6.3.1.320-326

Lucchi, N.W., Komino, F., Okoth, S.A., Goldman, I., Onyona, P., Wiegand, R.E., Juma, E., Shi, Y.P., Barnwell, J.W., Udhayakumar, V., & Kariuki, S. (2015). In Vitro and molecular surveillance for antimalarial drug resistance in *Plasmodium falciparum* parasites in Western Kenya reveals sustained artemisinin sensitivity and increased chloroquine sensitivity. *Antimicrobial Agents and Chemotherapy*, *59*(12), 7540-7547. https://doi.org/10.1128/AAC.01894-15

Maiga, H., Grivoyannis, A., Sagara, I., Traore, K., Traore, O.B., Tolo, Y., Traore, A., Bamadio, A., Traore, Z. I., Sanogo, K., Doumbo, O.K., Plowe, C.V., & Djimde, A.A. (2021). Selection of *Pfcrt* K76 and *Pfmdr1* N86 coding alleles after uncomplicated malaria treatment by artemether-lumefantrine in Mali. *International Journal of Molecular Sciences*, *22*(6057). https://doi.org/10.3390/ijms22116057

Malmberg, M., Ferreira, P.E., Tarning, J., Ursing, J., Ngasala, B., Björkman, A., Mårtensson, A., & Gil, J. P. (2013). *Plasmodium falciparum* drug resistance phenotype as assessed by patient antimalarial drug levels and its association with *Pfmdr1* polymorphisms. *Journal of Infectious Diseases*, *207*(5), 842-847. https://doi.org/10.1093/infdis/jis747

Massebo, F., Balkew, M., Gebre-Michael, T., & Lindtjorn, B. (2013). Blood meal origins and insecticide susceptibility of *Anopheles arabiensis* from Chano in Southwest Ethiopia. *Parasites & Vectors*, *6*(44). https://doi.org/10.1186/1756-3305-6-44

Mayxay, M., Pukrittayakamee, S., Chotivanich, K., Imwong, M., Looareesuwan, S., & White, N. J. (2001). Identification of cryptic coinfection with *Plasmodium falciparum* in patients presenting with vivax malaria. *American Journal of Tropical Medicine and Hygiene*, *65*(5), 588-592. https://doi.org/10.4269/ajtmh.2001.65.588

Mbanefo, A., & Kumar, N. (2020). Evaluation of malaria diagnostic methods as a key for successful control and elimination programs. *Tropical Medicine and Infectious Disease*, *5*(102). https://doi.org/10.3390/tropicalmed5020102

Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R.V., Rizk, S.S., Njimoh, D.L., Ryan, Y., Chotivanich, K., Nguon, C., Ghorbal, M., Lopez-Rubio, J.J., Pfrender, M., Emrich, S., Mohandas, N., Dondorp, A.M., Wiest, O., & Haldar, K. (2015). A molecular mechanism of artemisinin resistance in *Plasmodium falciparum* malaria. *Nature*, *520*, 683-687. https://doi.org/10.1038/nature14412

Mehlotra, R., Mattera, G., Bockarie, M., Maguire, J., Baird, J., Sharma, Y., Alifrangis, M., Dorsey, G., Rosenthal, P., Fryauff, D., Kazura, J., Stoneking, M., & Zimmerman, P. (2008). Discordant patterns of genetic variation at two chloroquine resistance loci in worldwide populations of the malaria parasite *Plasmodium falciparum*. *Antimicrobial Agents & Chemotherapy*, *52*(6), 2212-2222. https://doi.org/10.1128/AAC.00089-08

Meibalan, E., & Marti, M. (2017). Biology of malaria transmission. *Cold Spring Harb Perspectives in Medicine*, *7*. https://doi.org/10.1101/cshperspect.a025452

Mekonnen, S.K., Aseffa, A., Berhe, N., Teklehaymanot, T., Clouse, R.M., Gebru, T., Medhin, G., & Velavan, T.P. (2014). Return of chloroquine-sensitive *Plasmodium falciparum* parasites and emergence of chloroquine-resistant *Plasmodium vivax* in Ethiopia. *Malaria Journal*, *13*(244). https://doi.org/10.1186/1475-2875-13-244

Menegon, M., Nurahmed, A.M., Talha, A.A., Nour, B.Y.M., & Severini, C. (2016). Molecular surveillance of antimalarial drug resistance related genes in *Plasmodium falciparum*

isolates from Eritrea. *Acta Tropica*, *157*, 158-161. DOI:10.1016/j.actatropica.2016.02.007

Mitri, C., Thiery, I., Bourgouin, C., & Paul, R.E.L. (2009). Density-dependent impact of the human malaria parasite *Plasmodium falciparum* gametocyte sex ratio on mosquito infection rates. *Proceedings of the Royal Society Biological Sciences*, *276*, 3721-3726. https://doi.org/10.1098/rspb.2009.0962

Miura, K., Deng, B., Tullo, G., Diouf, A., Moretz, S.E., Locke, E., Morin, M., Fay, M.P., & Long, C. A. (2013). Qualification of standard membrane-feeding assay with P*lasmodium falciparum* malaria and potential improvements for future assays. *PLoS ONE*, *8*(3). https://doi.org/10.1371/journal.pone.0057909

Miura, K., Stone, W.J.R., Koolen, K.M., Deng, B., Zhou, L., Van Gemert, G.J., Locke, E., Morin, M., Bousema, T., Sauerwein, R.W., Long, C.A., & Dechering, K.J. (2016). An inter-laboratory comparison of standard membrane-feeding assays for evaluation of malaria transmission-blocking vaccines. *Malaria Journal*, *15*(463). 10.1186/s12936-016-1515-z

MOH. (2012). Ministry of Health, National Malaria guidelines. Addis Ababa, Ethiopia.

MOH. (2014). Ministry of Health, Disease Prevention and Control Directorate , Malaria Control Programme. Addis Ababa, Ethiopia.

MOH. (2017). Ministry of Health, National Malaria Elimination Roadmap. Addis Ababa, Ethiopia.

MOH. (2020). Ministry of Health, Ethiopia Malaria Elimination Strategic Plan: 2021-2025. Addis Ababa, Ethiopia.

Mohammed, A., Ndaro, A., Kalinga, A., Manjurano, A., Mosha, J. F., Mosha, D. F., Van Zwetselaar, M., Koenderink, J. B., Mosha, F. W., Alifrangis, M., Reyburn, H., Roper, C., & Kavishe, R. A. (2013). Trends in chloroquine resistance marker, *Pfcrt*-K76T mutation ten years after chloroquine withdrawal in Tanzania. *Malaria Journal*, *12*(415). https://doi.org/10.1186/1475-2875-12-415

Mohon, A.N., Getie, S., Jahan, N., Alam, M.S., & Pillai, D.R. (2019). Ultrasensitive loop mediated isothermal amplification (US-LAMP) to detect malaria for elimination. *Malaria*

Journal, *18*(350). https://doi.org/10.1186/s12936-019-2979-4

Morris, U., Khamis, M., Aydin-schmidt, B., Abass, A.K., Msellem, M.I., Nassor, M.H., González, I.J., Mårtensson, A., Ali, A.S., Björkman, A., & Cook, J. (2015). Field deployment of loop-mediated isothermal amplification for centralized mass-screening of asymptomatic malaria in Zanzibar : A pre-elimination setting. *Malaria Journal*, *14*(205). https://doi.org/10.1186/s12936-015-0731-2

Mula, P., Fernández-Martínez, A., De Lucio, A., Ramos, J., Reyes, F., González, V., Benito, A., & Berzosa, P. (2011). Detection of high levels of mutations involved in anti-malarial drug resistance in *Plasmodium falciparum* and *Plasmodium vivax* at a rural hospital in Southern Ethiopia. *Malaria Journal*, *10*(214). https://doi.org/10.1186/1475-2875-10-214

Mulenga, M.C., Sitali, L., Ciubotariu, I.I., Hawela, M.B., & Hamainza, B. (2021). Decreased prevalence of the *Plasmodium falciparum Pfcrt* K76T and *Pfmdr1* and N86Y mutations post-chloroquine treatment withdrawal in Katete District, Eastern Zambia. *Malaria Journal*, *20*(329). https://doi.org/10.1186/s12936-021-03859-z

Murmu, L., Sahu, A., & Barik, K. (2021). Diagnosing the drug resistance signature in *Plasmodium falciparum* : A review from contemporary methods to novel approaches. *Journal of Parasitic Diseases*, *45*, 869-876. https://doi.org/10.1007/s12639-020-01333-2.

Musiime, A., Okoth, J., Conrad, M., Ayo, D., Onyige, I., Rek, J., Nankabirwa, J., Arinaitwe, E., Kamya, M., Dorsey, G., Gemert, G., Staedke, S., Drakeley, C., Bousema, T., & Andolina, C. (2019). Is that a real oocyst ? Insectary establishment and identification of *Plasmodium falciparum* oocysts in midguts of *Anopheles* mosquitoes fed on infected human blood in Tororo, Uganda. *Malaria Journal*, *18*(287). https://doi.org/10.1186/s12936-019-2922-8

Mutero, C.M., Okoyo, C., Girma, M., Mwangangi, J., Kibe, L., Ng, P., Kussa, D., Diiro, G., Affognon, H., & Mbogo, C.M. (2020). Evaluating the impact of larviciding with *Bti* and community education and mobilization as supplementary integrated vector management interventions for malaria control in Kenya and Ethiopia. *Malaria Journal*, *19*(390). https://doi.org/10.1186/s12936-020-03464-6

Mwai, L., Kiara, S.M., Abdirahman, A., Pole, L., Rippert, A., Diriye, A., Bull, P., Marsh, K., Borrmann, S., & Nzila, A. (2009). In vitro activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan *Plasmodium falciparum* isolates and polymorphisms in *Pfcrt* and *Pfmdr1*. *Antimicrobial Agents & Chemotherapy*, *53*(12), 5069-5073. https://doi.org/10.1128/AAC.00638-09

Mwaiswelo, R.O., Ngasala, B., Msolo, D., Kweka, E., Mmbando, B.P., & Mårtensson, A. (2022). A single low dose of primaquine is safe and sufficient to reduce transmission of *Plasmodium falciparum* gametocytes regardless of cytochrome P450 2D6 enzyme activity in Bagamoyo district, Tanzania. *Malaria Journal*, *21*(84). https://doi.org/10.1186/s12936-022-04100-1

Mwangi T.W., Ross A, Snow R.W., & Marsh K. (2005). Case definitions of clinical malaria under different transmission conditions in Kilifi District, Kenya. *Journal of Infectious Diseases, 191*, 1932-1939. https://doi.org/10.1086/430006

Mwanza, S., Joshi, S., Nambozi, M., Chileshe, J., Malunga, P., Kabuya, J.B.B., Hachizovu, S., Manyando, C., Mulenga, M., & Laufer, M. (2016). The return of chloroquine-susceptible *Plasmodium falciparum* malaria in Zambia. *Malaria Journal*, *15*(584). https://doi.org/10.1186/s12936-016-1637-3

Ndo, C., Kopya, E., Menze-Djantio, B., Toto, J.C., Awono-Ambene, P., Lycett, G., & Wondji, C.S. (2016). High susceptibility of wild *Anopheles funestus* to infection with natural *Plasmodium falciparum* gametocytes using membrane feeding assays. *Parasites and Vectors*, *9*(341). https://doi.org/10.1186/s13071-016-1626-y

Nega, D., Assefa, A., Mohamed, H., & Solomon, H. (2016). Therapeutic efficacy of artemether- lumefantrine (Coartem®) in treating uncomplicated *Plasmodium falciparum* malaria in metehara, Eastern Ethiopia : regulatory clinical study. *PlOS ONE*, *11*(4). https://doi.org/10.1371/journal.pone.0154618

Nega, D., Daniel, D., Tefera, T. & Eshetu, T. (2015). Prevalence and predictors of asymptomatic malaria parasitemia among pregnant women in the rural surroundings of Arbaminch town. *PLoS ONE*, *10*(4). https://doi.org/10.1371/journal.pone.0123630

Nilsson, S.K., Childs, L.M., Buckee, C., & Marti, M. (2015). Targeting human transmission biology for malaria elimination. *PLoS Pathogens*, *11*(6). DOI:10.1371/journal.ppat.1004871

Nina, B.P., Mohanty, A.K., Ballav, S., Vernekar, S., Bhinge, S., D'Souza, M., Walke, J., Manoharan, S.K., Mascarenhas, A., Gomes, E., Chery, L., Valecha, N., Kumar, A., & Rathod, P. K. (2017). Dynamics of *Plasmodium vivax* sporogony in wild *Anopheles stephensi* in a malaria-endemic region of Western India. *Malaria Journal*, *16*(284). https://doi.org/10.1186/s12936-017-1931-8.

Njiro, B.J., Mutagonda, R.F., Chamani, A.T., Mwakyandile, T., Sabas, D., & Bwire, G.M. (2022). Molecular surveillance of chloroquine-resistant *Plasmodium falciparum* in sub-Saharan African countries after withdrawal of chloroquine for treatment of uncomplicated malaria: A systematic review. *Journal of Infection and Public Health*, *15*(5), 550-557. https://doi.org/10.1016/j.jiph.2022.03.015

Nsanzabana, C., Djalle, D., Guérin, P.J., Ménard, D., & González, I.J. (2018). Tools for surveillance of anti-malarial drug resistance : An assessment of the current landscape. *Malaria Journal*, *17*(75). https://doi.org/10.1186/s12936-018-2185-9

Nureye, D., & Assefa, S. (2020). Old and recent advances in life cycle, pathogenesis, diagnosis, prevention, and treatment of malaria including perspectives in Ethiopia. *The Scientific World Journal*, *2020*(1295381). https://doi.org/10.1155/2020/1295381

Nyataya, J., Waitumbi, J., Mobegi, V.A., Zowalaty, M.E.E. (2020). *Plasmodium falciparum* histidine-rich protein 2 and 3 gene deletions and their implications in malaria control. *Diseases*, *8*(15). https://doi.org/doi:10.3390/diseases8020015

O'Neill, P.M., Barton, V.E., & Ward, S.A. (2010). The molecular mechanism of action of artemisinin: the debate continues. *Molecules*, *15*(3), 1705-1721. https://doi.org/10.3390/molecules15031705

Ocan, M., Akena, D., Nsobya, S., Kamya, M.R., Senono, R., Kinengyere, A.A., & Obuku, E. A. (2019). Persistence of chloroquine resistance alleles in malaria endemic countries : A systematic review of burden and risk factors. *Malaria Journal*, *18*(76).

https://doi.org/10.1186/s12936-019-2716-z

Okell, L.C., Reiter, L.M., Ebbe, L.S., Baraka, V., Bisanzio, D., Watson, O.J., Bennett, A., Verity, R., Gething, P., Roper, C., & Alifrangis, M. (2018). Emerging implications of policies on malaria treatment: Genetic changes in the *Pfmdr-1* gene affecting susceptibility to artemether–lumefantrine and artesunate–amodiaquine in Africa. *BMJ Global Health*, *3*. https://doi.org/10.1136/bmjgh-2018-000999

Otienoburu, S.D., Maïga-Ascofaré, O., Schramm, B., Jullien, V., Jones, J.J., Zolia, Y.M., Houzé, P., Ashley, E. A., Kiechel, J. R., Guérin, P. J., Le Bras, J., & Houzé, S. (2016). Selection of *Plasmodium falciparum Pfcrt* and *Pfmdr1* polymorphisms after treatment with artesunate-amodiaquine fixed dose combination or artemether-lumefantrine in Liberia. *Malaria Journal*, *15*(452). https://doi.org/10.1186/s12936-016-1503-3

Ouédraogo, A.L., Bousema, T., Schneider, P., de Vlas, S.J., Ilboudo-Sanogo, E., Cuzin-Ouattara, N., Nábí, I., Roeffen, W., Verhave, J.P., Luty, A.J.F., & Sauerwein, R. (2009). Substantial contribution of submicroscopical *Plasmodium falciparum* gametocyte carriage to the infectious reservoir in an area of seasonal transmission. *PLoS ONE*, *4*(12). https://doi.org/10.1371/journal.pone.0008410

Ouédraogo, A.L., Gonçalves, B.P., Gnémé, A., Wenger, E.A., Guelbeogo, M.W., Ouédraogo, A., Gerardin, J., Bever, C.A., Lyons, H., Pitroipa, X., Verhave, J.P., Eckhoff, P.A., Drakeley, C., Sauerwein, R., Luty, A.J.F., Kouyaté, B., & Bousema, T. (2016). Dynamics of the human infectious reservoir for malaria determined by mosquito feeding assays and ultrasensitive malaria diagnosis in Burkina Faso. *Journal of Infectious Diseases*, *213*(1), 90-99. https://doi.org/10.1093/infdis/jiv370

Ouédraogo, A.L., Guelbéogo, W.M., Cohuet, A., Morlais, I., King, J.G., Gonçalves, B.P., Bastiaens, G.J.H., Vaanhold, M., Sattabongkot, J., Wu, Y., Coulibaly, M., Ibrahima, B., Jones, S., Morin, M., Drakeley, C., Dinglasan, R.R., & Bousema, T. (2013). A protocol for membrane feeding assays to determine the infectiousness of *Plasmodium falciparum* naturally infected individuals to *Anopheles gambiae*. *Malaria World Journal*, *4*(16), 17-20. ISSN 2214-4374.

Petersen, I., Eastman, R., & Lanzer, M. (2011). Drug-resistant malaria: molecular mechanisms and implications for public health. *FEBS Letters*, *585*(11), 1551-1562. https://doi.org/10.1016/j.febslet.2011.04.042

Plowe, C. V. (2003). Monitoring antimalarial drug resistance: making the most of the tools at hand, *Journal of Experimental Biology*, *206*(21), 3745-3752. DOI: 10.1242/jeb.00658

Raghavendra, K., & Barik, T.K. (2011). Malaria vector control: From past to future. *Parasitology and Resistance*, *108*, 757-779. https://doi.org/10.1007/s00436-010-2232-0

Reed, M.B., Saliba, K. J., Caruana, S.R., Kirk, K., & Cowman, A.F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature*, *403*, 906-909. https://doi.org/10.1038/35002615

Riche T.L. & Saul A. (2002). Progress and challenges for malaria vaccines. *Nature*, *415*, 694-701. https://doi.org/10.1038/415694a

Robert, V., Sokhna, C.S., Rogier, C., Ariey, F., & Trape, J.F. (2003). Sex ratio of *Plasmodium falciparum* gametocytes in inhabitants of Dielmo, Senegal. *Parasitology*, *127*. https://doi.org/10.1017/S0031182003003299

Rosenthal, P. (2022). Editorial malaria in 2022 : challenges and progress. *American Journal of Tropical Medicine and Hygein*, *106*(6), 1565-1567. https://doi.org/10.4269/ajtmh.22-0128

Rout, S., & Mahapatra, R.K. (2019). *Plasmodium falciparum*: multidrug resistance. *Chemical Biology and Drug Design*, *93*, 737-759. https://doi.org/10.1111/cbdd.13484

Roux, A.T., Maharaj, L., Oyegoke, O., Akoniyon, O.P., Adeleke, M.A., Maharaj, R., & Okpeku, M. (2021). Chloroquine and sulfadoxine-pyrimethamine resistance in sub-Saharan Africa: A review. *Frontiers in Genetics*, *12*. https://doi/10.3389/fgene.2021.668574

Sahoo, P.R., Sethy, K., Mohapatra, S., & Panda, D. (2016). Loop mediated isothermal amplification: An innovative gene amplification technique for animal diseases. *Veterinary World*, *9*(5), 465-469. https://doi.org/10.14202/vetworld.2016.465-469

Saifi, A., Beg, T., Harrath, A.H., Altayalan, F.S.H., & Quraishy, S. (2013). Antimalarial drugs: mode of action and status of resistance. *African Journal of Pharmacy and Pharmacology*, *7*(5), 148-156. https://doi.org/10.5897/ajppx12.015

Sanchez, C.P., Dave, A., Stein, W.D., & Lanzer, M. (2010). Transporters as mediators of drug resistance in *Plasmodium falciparum*. *International Journal for Parasitology*, *40*, 1109-1118. https://doi.org/10.1016/j.ijpara.2010.04.001

Sanchez, C.P., Rohrbach, P., McLean, J.E., Fidock, D.A., Stein, W.D., & Lanzer, M. (2007). Differences in trans-stimulated chloroquine efflux kinetics are linked to PfCRT in *Plasmodium falciparum*. *Molecular Microbiology*, *64*(2), 407-420.

Santos, N. A.C., Andrade, A.O., Santos, T.C., Martinez, L.N., Ferreira, A.S., Bastos, A.S., Martins, M.M., Pontual, J.D.C., Teles, C.B.G., Medeiros, J.F., & Araújo, M.S. (2022). Evaluation of sustainable susceptibility to *Plasmodium vivax* infection among colonized *Anopheles darlingi* and *Anopheles deaneorum*. *Malaria Journal*, *21*(163). https://doi.org/10.1186/s12936-022-04204-8

Sato, S. (2021). *Plasmodium*: A brief introduction to the parasites causing human malaria and their basic biology. *Journal of Phsiological Anthropology*, *40*(1). https://doi.org/10.1186/s40101-020-00251-9.

Sattabongkot, J., Kumpitak, C., & Kirakorn, K. (2015). *Membrane feeding assay to determine the infectiousness of Plasmodium vivax gametocytes*. S. A. Vaughan (Ed.). *Malaria Vaccines Methods and Protocols* (pp. 93-99). New York, NY: Human Press.

Schneider, P., Bousema, T., Omar, S., Gouagna, L., Sawa, P., Schallig, H., & Sauerwein, R. (2006). (Sub) microscopic *Plasmodium falciparum* gametocytaemia in Kenyan children after treatment with sulphadoxine-pyrimethamine monotherapy or in combination with artesunate. *International Journal for Parasitology*, *36*, 403-408. https://doi.org/10.1016/j.ijpara.2006.01.002

Schneider, P., Teun, B.J., Louis, C.G., Silas, O., Vegte-Bolmer R,M. Van de, Omar, A.S., & Sauerwein, A.W. (2007). Submicroscopic *Plasmodium falciparum* gametocyte densities. *American Journal of Tropical Medicine and Hygein, 76*(3), 470-474. ISSN 0002-9637

Schunk, M., Kumma, W.P., Miranda, I.B., Osman, M.E., Roewer, S., Alano, A., Löscher, T., Bienzle, U., & Mockenhaupt, F.P. (2006). High prevalence of drug-resistance mutations in *Plasmodium falciparum* and *Plasmodium vivax* in Southern Ethiopia. *Malaria Journal*, *5*(54). https://doi.org/10.1186/1475-2875-5-54

Service, W., & Townson, H. (2002). *The Anopheles Vector*. H. Gilles and D. Warrell (Eds.). *Essential Malariology* (4th Ed., pp. 59-84), Arnold, London, UK: CRC Press.

Setyaningrum, E., Santoso, B., A,R., & Suryaningkunti, J.A. (2021). The potential of temephos as larvacide for malaria vector control in the potential of temephos as larvacide for malaria vector control in katibung sub-district, South Lampung. *Journal of Physics: Conference Series*, *1751*(012056). https://doi.org/10.1088/1742-6596/1751/1/012056.

Shafik, S.H., Cobbold, S.A., Barkat, K., Richards, S.N., Lancaster, N.S., Llinás, M., Hogg, S. J., Summers, R.L., McConville, M.J., & Martin, R.E. (2020). The natural function of the malaria parasite's chloroquine resistance transporter. *Nature Communications*, *11*(3922). https://doi.org/10.1038/s41467-020-17781-6

Shandilya, A., Chacko, S., Jayaram, B., & Ghosh, I. (2013). A plausible mechanism for the antimalarial activity of artemisinin: A computational approach. *Scientific Reports*, *3*(2513). https://doi.org/10.1038/srep02513

Shrivastava, S.K., Gupta, R.K., Mahanta, J., & Dubey, M.L. (2014). Correlation of molecular markers, *Pfmdr1*-N86Y and *Pfcrt*-K76T, with *in vitro* chloroquine resistant *Plasmodium falciparum*, isolated in the malaria endemic states of Assam and Arunachal Pradesh, Northeast India. *PlOS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0103848

Sinka, M.E., Pironon, S., Massey, N.C., Longbottom, J., Hemingway, J., Moyes, C.L., & Willis, K.J. (2020). A new malaria vector in Africa : Predicting the expansion range of *Anopheles stephensi* and identifying the urban populations at risk. *Proceedings of National Academy of Sciences*, *117*(40), 24900-24908. https://doi.org/10.1073/pnas.2003976117

Sisowath, C., Ferreira, P.E., Bustamante, L.Y., Dahlström, S., Mårtensson, A., Björkman, A., Krishna, S., & Gil, J.P. (2007). The role of *Pfmdr1* in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. *Tropical Medicine and International Health*, *12*(6),

736-742. https://doi.org/10.1111/j.1365-3156.2007.01843.x

Sisowath, C., Petersen, I., Veiga, M.I., Mårtensson, A., Premji, Z., Björkman, A., Fidock, D. A., & Gil, J.P. (2009). *In Vivo* selection of *Plasmodium falciparum* parasites carrying the chloroquine-susceptible *Pfcrt* K76 allele after treatment with artemether-lumefantrine in Africa. *Journal of Infectious Diseases*, *199*(5), 750-757. https://doi.org/10.1086/596738

Sisowath, C., Strömberg, J., Mårtensson, A., Msellem, M., Obondo, C., Björkman, A., & Gil, J. P. (2005). *In vivo* selection of *Plasmodium falciparum Pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem®). *Journal of Infectious Diseases*, *191*(6), 1014-1017, https://doi.org/10.1086/427997

Solarte, Y., Manzano, M.R., Rocha, L., Hurtado, H., James, M.A., Arévalo-Herrera, M., & Herrera, S. (2011). *Plasmodium vivax* sporozoite production in *Anopheles albimanus* mosquitoes for vaccine clinical trials. *American Journal of Tropical Medicine and Hygiene*, *84*(2), 28-34. https://doi.org/10.4269/ajtmh.2011.09-0499

Sondo, P., Derra, K., Nakanabo, S.D., Tarnagda, Z., Kazienga, A., Zampa, O., Valéa, I., Sorgho, H., Owusu-Dabo, E., Ouédraogo, J.B., Guiguemdé, T.R., & Tinto, H. (2016). Artesunate-amodiaquine and artemether-lumefantrine therapies and selection of *Pfcrt* and *Pfmdr1* alleles in Nanoro, Burkina Faso. *PLoS ONE*, *11*(3). https://doi.org/10.1371/journal.pone.0151565.

Sougoufara, S., Ottih, E.C., & Tripet, F. (2020). The need for new vector control approaches targeting outdoor biting anopheline malaria vector communities. *Parasites & Vectors*, *13*(295). https://doi.org/10.1186/s13071-020-04170-7

Soumare, H.M., Guelbeogo, W.M., Bolmer, M. V.D.V., Gemert, G.J.V., Soumanaba, Z., Ouedraogo, A., Ouattara, M.S., Abdullahi, A., Jadama, L., Camara, M.M., Gaye, P.M., Mendy, M., Davis, N., Tiono, A.B., Alessandro, U.D., Drakeley, C., Bousema, T., Moreno, M., & Collins, K.A. (2021). Maintaining *Plasmodium falciparum* gametocyte infectivity during blood collection and transport for mosquito feeding assays in the field. *Malaria Journal*, *20*(191). https://doi.org/10.1186/s12936-021-03725-y

Stone, W.J.R., Campo, J.J., Ouédraogo, A.L., Meerstein-Kessel, L., Morlais, I., Da, D., Cohuet, A., Nsango, S., Sutherland, C.J., Van De Vegte-Bolmer, M., Siebelink-Stoter, R., Van Gemert, G.J., Graumans, W., Lanke, K., Shandling, A.D., Pablo, J.V., Teng, A.A., Jones, S., De Jong, R.M.,…Jore, M.M. (2018). Unravelling the immune signature of *Plasmodium falciparum* transmission-reducing immunity. *Nature Communications*, *9*(558). https://doi.org/10.1038/s41467-017-02646-2

Stone, W.J.R., Eldering, M., van Gemert,G.J., Lanke,K.H.W., Grignard, L., Van De Vegte-Bolmer, M., Siebelink-Stoter, R., Graumans, W., Roeffen, W.F., Drakeley, Ch.J., Sauerwein, R.W. & Bousema, T. (2013). The relevance and applicability of oocyst prevalence as a read-out for mosquito feeding assays. *Scientific Report*, *3*(3418). https://doi.org/ 10.1038/srep03418.

Stone, W., Sawa, P., Lanke, K., Rijpma, S., Oriango, R., Nyaurah, M., Osodo, P., Osoti, V., Mahamar, A., Diawara, H., Woestenenk, R., Graumans, W., Van De Vegte-Bolmer, M., Bradley, J., Chen, I., Brown, J., Siciliano, G., Alano, P., Gosling, R., … Bousema, T. (2017). A molecular assay to quantify male and female *Plasmodium falciparum* gametocytes: Results from 2 randomized controlled trials using primaquine for gametocyte clearance. *Journal of Infectious Diseases*, *216*(4), 457-467. https://doi.org/10.1093/infdis/jix237

Tadele, G., Jaiteh, F.K., Oboh, M., Oriero, E., Dugassa, S., Amambua-Ngwa, A., & Golassa, L. (2022). Persistence of residual submicroscopic *Plasmodium falciparum* parasitemia following treatment of artemether-lumefantrine in Ethio-Sudan border, Western Ethiopia. *Antimicrobial Agents and Chemotherapy*, *20*(20). https://doi.org/10.1128/aac.00002-22

Tadesse, F.G., Ashine, T., Teka, H., Esayas, E., Messenger, L.A., Chali, W., Meerstein-kessel, L., Walker, T., Behaksra, S. W., Lanke, K., Heutink, R., Jeffries, C.L., Mekonnen, D.A., Hailemeskel, E., Tebeje, S.K., Tafesse, T., Gashaw, A., Tsegaye, T., Emiru, T.,…Bousema, T.(2021). *Anopheles stephensi* mosquitoes as vectors of *Plasmodium vivax* and *Plasmodium falciparum*. *Emerging Infectious Diseases*, *27*(2), 603-607. https://doi.org/10.5061/dryad.gf1vhhmnt

Tadesse, F.G., Slater, H.C., Chali, W., Teelen, K., Lanke, Kj., Belachew, M., Menberu, T., Shume, G., Shitaye, G., Okell, L.C., Graumans, W., Gemert, G.J.V., Kedir, S., Tesfaye A., Belachew F., Abebe, W., Mamo, H., Sauerwein, R., Balcha, T.,…Bousema, T. (2018). The relative contribution of symptomatic and asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections to the infectious reservoir in a low-endemic setting in Ethiopia. *Clinical Infectious Diseases*, *66*(12), 1883-1891. https://doi.org/10.1093/cid/cix1123

Taffese, H.S., Hemming-Schroeder, E., Koepfli, C., Tesfaye, G., Lee, M. ., Kazura, J., Yan, G. Y., & Zhou, G.F. (2018). Malaria epidemiology and interventions in Ethiopia from 2001 to 2016. *Infectious Diseases of Poverty*, *7*(103). https://doi.org/10.1186/s40249-018-0487-3

Tajebe, A., Aemero, M., Francis, K., & Magoma, G. (2015). Identification of chloroquine resistance *Pfcrt*-K76T and determination of *Pfmdr1*-N86Y copy number by SYBR Green I qPCR. *Asian Pacific Journal of Tropical Biomedicine*, *5*(3), 208-220. https://doi.org/10.1016/S2221-1691(15)30008-3

Tangpukdee, N., Duangdee, C., Wilairatana, P., & Krudsood, S. (2009). Malaria Diagnosis : A brief review. *Korean Journal of Parasitology*, *47*(2), 93-102. https://doi.org/10.3347/kjp.2009.47.2.93

Tarekegn, M., Wolde-hawariat, Y., Dugassa, S., & Tekie, H. (2021). Evaluation of larvicidal activities of *Parthenium hysterophorus* Liston against *Anopheles arabiensis* (Diptera : Culicidae), the major malaria vector in Ethiopia. *International Journal of Tropical Insect Science*, *41*, 1461-1469. https://doi.org/10.1007/s42690-020-00344-z

Taye, A., Hadis, M., Adugna, N., Tilahun, D., & Wirtz, R. A. (2006). Biting behavior and *Plasmodium* infection rates of *Anopheles arabiensis* from Sille, Ethiopia. *Acta Tropica*, *97*, 50-54, https://doi.org/10.1016/j.actatropica.2005.08.002

Tegegne, B., Getie, S., Lemma, W., Mohon, A.N., & Pillai, D. R. (2017). Performance of loop-mediated isothermal amplification (LAMP) for the diagnosis of malaria among malaria suspected pregnant women in Northwest Ethiopia. *Malaria Journal*, *16*(34). https://doi.org/10.1186/s12936-017-1692-4

Timinao, L., Vinit, R., Katusele, M., Koleala, T., Nate, E., Czeher, C., Burkot, T. R., Felger, I., Mueller, I., Laman, M., Robinson, L.J., & Karl, S. (2021). Infectivity of symptomatic malaria patients to *Anopheles farauti* colony mosquitoes in Papua New Guinea. *Frontiers in Cellular and Infectious Microbiology*, *11*(771233). DOI:10.3389/fcimb.2021.771233

Timinao, L., Vinit, R., Katusele, M., Schofield, L., Burkot, T.R., & Karl, S. (2021). Optimization of the feeding rate of *Anopheles farauti* s.s. colony mosquitoes in direct membrane feeding assays. *Parasites & Vectors*, *14*(356). DOI:10.1186/s13071-021-04842-y

Tinto, H., Guekoun, L., Zongo, I., Guiguemde, R.T., D'Alessandro, U., & Oue´draogo, J.B. (2008). Short communication chloroquine-resistance molecular markers (*Pfcrt* T76 and *Pfmdr-1* Y86) and amodiaquine resistance in Burkina Faso. *Tropical Medicine and International Health*, *13*(2), 238-240. https://doi.org/10.1111/j.1365-3156.2007.01995.x

Tse, E.G., Korsik, M., & Todd, M.H. (2019). The past, present and future of anti-malarial medicines. *Malaria Journal*, *18*(93). https://doi.org/10.1186/s12936-019-2724-z.

Tuedom, A.G.B., Sarah-Matio, E.M., Moukoko, C.E.E., Feufack-Donfack, B.L., Maffo, C.N., Bayibeki, A.N., Awono-Ambene, H.P., Ayong, L., Berry, A., Abate, L., Morlais, I., & Nsango, S.E. (2021). Antimalarial drug resistance in the Central and Adamawa regions of Cameroon: Prevalence of mutations in *Plasmodium falciparum crt*, *Pfmdr1*, *Pfdhf*r and *Pfdhps* genes. *PLoS ONE*, *16*(8). https://doi.org/10.1371/journal.pone.0256343

Ukpe, I.S., Moonasar, D., Raman, J., Barnes, K.I., Baker, L., & Blumberg, L. (2013). Case management of malaria: treatment and chemoprophylaxis. *South African Medical Journal*, *103*(10), 793-798. https://doi.org/10.7196/SAMJ.7443

Vallejo, A.F., García, J., Amado-Garavito, A.B., Arévalo-Herrera, M., & Herrera, S. (2016). *Plasmodium vivax* gametocyte infectivity in sub-microscopic infections. *Malaria Journal*, *15*(48). https://doi.org/10.1186/s12936-016-1104-1

Vallejo, A.F., Rubiano, K., Amado,A., & Krystosik, A.R. (2016). Optimization of a membrane feeding assay for *Plasmodium vivax* infection in *Anopheles albimanus*. *PLOS ONE*, *10*(6). https://doi.org/10.1371/journal.pntd.0004807

van Loon, W., Bergmann, C., Habarugira, F., Tacoli, C., Savelsberg, D., & Oliveira, R. (2021). Changing pattern of *Plasmodium falciparum Pfmdr1* gene polymorphisms in Southern Rwanda. *Antimicrobial Agents and Chemotherapy*, *65*(9). https://doi.org/10.1128/AAC.00901-21

Veiga, M.I., Dhingra, S.K., Henrich, P.P., Straimer, J., Gna, N., Uhlemann, A.C., Martin, R.E., Lehane A.N. & Fidock, D. (2016). Globally prevalent PfMDR1 mutations modulate *Plasmodium falciparum* susceptibility to artemisinin-based combination therapies. *Nature Communication, 7*(115530). https://doi.org/10.1038/ncomms11553

Venkatesan, M., Gadalla, N.B., Stepniewska, K., Dahal, P., Nsanzabana, C., Price, R.N., Mårtensson, A., Rosenthal, P.J., Dorsey, G., Sutherland, C. ., Guérin, P., Davis, T.M.E., Ménard, D., Adam, I., Ademowo, G., Arze, C., Baliraine, F.N., Berens-Richa, N.,… Sibley, H. (2014). Polymorphisms in *Plasmodium falciparum chloroquine resistance transporter* and *multidrug resistance 1* genes : Parasite risk factors that affect treatment outcomes for *Plasmodium falciparum* malaria after artemether-lumefantrine and artesunate-amodiaquine. *American Society of Tropical Medicine and Hygiene*, *91*, 833-843. https://doi.org/10.4269/ajtmh.14-0031

Wang, C.Y.T., Ballard, E., Llewellyn, S., Marquart, L., Bousema, T., Mccarthy, J.S., & Collins, K.A. (2020). Assays for quantification of male and female gametocytes in human blood by qRT-PCR in the absence of pure sex-specific gametocyte standards. *Malaria Journal*, *19*(218). https://doi.org/10.1186/s12936-020-03291-9.

White, N.J. (2011). Determinants of relapse periodicity in *Plasmodium vivax* malaria. *Malaria Journal*, *10*(297). https://doi.org/10.1186/1475-2875-10-297

White, N J., Pukrittayakamee, S., Hien, T.T., Faiz, M.A., Mokuolu, O.A., & Dondorp, A.M. (2013). Malaria. *The Lancet*, 383, 723-735. https://doi.org/10.1016/S0140-6736(13)60024-0

WHO (2008a). Global Malaria Control and Elimination: Malaria Control and Elimination : World Health Organization, World Health Organization, *Geneva, Switzerland.*

WHO (2008b). World Malaria Report 2008. World Health Organization, Geneva, Switzerland.

WHO (2009). Methods for surveillance of antimalarial drug efficacy. World Health Organization, Geneva, Switherland.

WHO (2010a). World Malaria Report 2010, World Health Organization, Geneva, Switzerland.

WHO (2010b). Global report on ant-malarial drug efficacy and drug resistance:2000-2010. World Health Organization, Geneva, Switzerland

WHO (2010c). Basic Malaria Microscopy. *Part I. Learner's guide, 2nd edition*. World Health Organization, Geneva, Switzerland.

WHO (2015). Giudelines for the treatment of malaria. 3rd edition. World Health Organization, Geneva, Switzerland.

WHO (2016a). Global report on insecticide resistance in malaria vectors: 2010-2016. World Health Organization, Geneva, Switzerland.

WHO (2016b). Global technical strategy for malaria 2016–2030. World Health Organization, Geneva, Switzerland.

WHO (2017). Status report on Artemisinin and ACT resistance (April 2017). World Health Organization. World Health Organization, Geneva, Switzerland.

WHO (2020). World malaria report 2020. World Health Organization, Geneva, Switzerland.

WHO (2022). World Malaria Report 2022. World Health Organization, Geneva, Switzerland.

WHO (2021a). WHO Guidelines for malaria treatment 2021. World Health Organization, Geneva, Switzerland.

WHO (2021b). WHO recommends groundbreaking malaria vaccine for children at risk. World Health Organization, Geneva, Switzerland.

Wicht, K.J., Mok, S., & Fidock, D.A. (2020). Molecular mechanisms of drug resistance in *Plasmodium falciparum* malaria. *Annual Review of Microbiology*, *74*, 431-454, https://doi.org/org/10.1146/annurev-micro-020518-115546

Witkowski, B., Amaratunga, C., Khim, N., Sreng, S., Chim, P., Kim, S., Lim, P., Mao, S., Sopha, C., Sam, B., Anderson, J.M., Duong, S., Chuor, C.M., Taylor, W.R.J., Suon, S., Mercereau-Puijalon, O., Fairhurst, R.M., & Menard, D. (2013). Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: *In vitro* and *ex vivo* drug-response studies. *The Lancet Infectious Diseases*, *13*(12), 1043-1049. https://doi.org/10.1016/S1473-3099(13)70252-4

Woodrow, C.J., Dahlström, S., Cooksey, R., Flegg, J.A., Nagard, L., Mentré, F., & Murillo, C. (2013). High-throughput analysis of antimalarial susceptibility data by the Worldwide antimalarial resistance network (WWARN) *In Vitro*. *Antimicrobial Agents & Chemotherapy*, *57*(7), 3121-3130. https://doi.org/10.1128/AAC.02350-12

Wurtz, N., Fall, M., Baret, E., Camara, C., Nakoulima, A., Diatta, B., Fall, K.B., Mbaye, P.S., Bercion, R., Wade, B., & Pradines, B. (2014). Role of *Pfmdr1* in in vitro *Plasmodium falciparum* susceptibility to chloroquine, quinine, monodesethylamodiaquine, mefloquine, lumefantrine and dihydroartemisini. *Antimicrobial Agents & Chemotherapy*, *58*(12), 7032-7040, https://doi.org/10.1128/AAC.03494-14

Yobi, D.M., Kayiba, N.K., Mvumbi, D.M., Boreux, R., Kabututu1, P.Z., Situakibanza, H.N.T., Likwela, J.L., Moe, P.D., Okitolonda, E.W., Niko Speybroeck, N., Mvumbi, G.L. & Hayette, M.P. (2020). Molecular surveillance of anti-malarial drug resistance in Democratic Republic of Congo: high variability of chloroquinoresistance and lack of amodiaquinoresistance. *Malaria Journal*, *19*(121). https://doi.org/10.1186/s12936-020-03192-x

Yongkiettrakul, S., Kolié, F.R., Kongkasuriyachai, D., Sattabongkot, J., Nguitragool, W., Nawattanapaibool, N., Suansomjit, C., Warit, S., Kangwanrangsan, N., & Buates, S. (2020). Validation of *Pf* SNP-LAMP-lateral flow dipstick for detection of single nucleotide polymorphism associated with pyrimethamine resistance in *Plasmodium falciparum*. *Diagnostics*, *10*(948). https://doi.org/10.3390/diagnostics10110948

Yu, Sh., Wang, J., Luo, X., Zheng, H., Wang, L., Yang, X., & Wang, Y. (2022). Transmission-blocking strategies against malaria parasites during their mosquito stages. *Frontiers in Cellular and Infection Microbiology*, *12*(820650).

https://doi.10.3389/fcimb.2022.820650

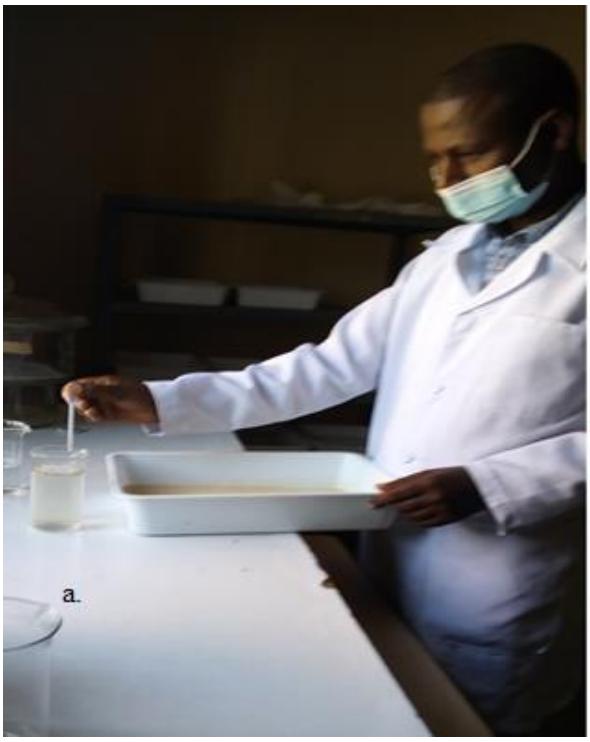
Zheng, Z., & Cheng, Z. (2017). Advances in molecular diagnosis of malaria. *Advances in Clinical Chemistry*, *80*, 155-192. http://dx.doi.org/10.1016/bs.acc.2016.11.006

Zhou, W., Wang, H., Yang, Y., Chen, Z.S., Zou, C., & Zhang, J. (2020). Chloroquine against malaria, cancers and viral diseases. *Drug Discovery Today*, *25*, 2012-2022, https://doi.org/10.1016/j.drudis.2020.09.010

APPENDICES

Appendix 1. Mosquito feeding on rabbit in the insectary.

Appendix 2. Rearing mosquitoes to be emerged in to adults.



Appendix 3. Process of direct membrane feeding assay.

Appendix 4. Blood fed mosquitoes kept in a separate room.

Appendix 5. Ethical clearance

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

OFFICE OF RESEARCH AFFAIRS

University Ethical Review Board (UERB)

Phone:+251-222-110780

Fax:+251-221-11048

Email: raff@astu.edu.et / alemu.disasa@astu.edu.et

CERTIFICATE OF APPROVAL

To: Jifar Hassen Mudi

Department: Applied Biology

Program: PhD

Study Title: Field-based Molecular Diagnosis of Low Density Asymptomatic *Plasmodium falciparum* Infections, Transmissibility and Molecular Marker Associated with Anti-malaria Drug Resistance in and around Adama City, Oromia, Ethiopia.

Approval Date: September 15, 2019

Certificate Ref. No: RECSoANS/BIO/02/2019

Based on your application for Ethical Clearance, the University Ethical Review Board (UERB) of ASTU has reviewed your dissertation proposal and approved within the framework to:

I. conduct the project in accordance with the approved dissertation proposal
II. immediately apply for any complaints in relation to your project which may warrant the review of the ethical approval record
III. provide progress report on your project at the end of every semester
IV. provide final report when the project is completed

Finally, I congratulate you for securing this Ethical Approval Certificate to precede your project.

Wishing you all the best!

Alemu Disassa Muleta (PhD)
Research Affairs Dean
Chairman

Appendix 6. Consent form for adults > 1 8years

Name: _____Age: _____Sex: _____

Identification No: _____Address: _____Date: _____

I have been well informed that Adama Science and Technology University and Aklilu Lemma Institute of Pathobiology Addis Ababa University would like to carry out a collaborative study on malaria entitled "**Molecular Epidemiology of Anti-malarial Drugs Resistance Markers in Clinical *Plasmodium falciparum* Isolates and Infectiousness of Symptomatic Patients to Mosquitoes in East Shewa Zone, Oromia Regional State, Ethiopia**". I am aware that the study requires about 5ml of the venous blood and asked if I agree to give these specimens with recognition of my right to withdraw from the study if I change my mind any time.

Please read, tick each of the boxes and sign if you agree to participate in the study

1. I understand what this study is about and know how to contact the investigators if I want to ☐
2. I understand the practical consequences of this study, asking me to provide small finger prick and 5ml of blood sample ☐
3. A portion of the blood may be stored and used for this study only. If there is left over after the completion of the study, it will be discarded safely. ☐
4. I understand a portion of blood sample may be analyzed outside Ethiopia ☐
5. I understand that all the information given to the investigators and all test results will be kept private and confidential. ☐
6. I understand that I am free to withdraw myself from this study if I want to. ☐
7. I understand that if I refuse to take part in this study, that my care will not be affected. ☐
8. I understand that results from this study will contribute to Network-wide research activities. ☐

I have been given enough time to think over before I signed this informed consent.

It is, therefore, with full understanding of the situation that I gave my informed consent to participate in this study.

Thumbprint if subject is unable to sign

The information was explained to me by _____

Participant's name: _____

Participant's signature: _____

Appendix 7. Proxy consent and assent for < 18 years

Name:_____Age:_____Sex:_____

IdentificationNo:_____Address:_____Date: _____

I, Mr/Mrs, _____being a person aged ≥ 18yrs and being the parent/Lawful guardian of my child (son/daughter) _____ hereby consent to Dr/Mr./Mrs./Miss _____ in the intended research as explained and understood by me.

I have understood the implications of risks and immediate benefits of the tests and treatment to my son/daughter _____. I have agreed my son/daughter _____to give 5ml venous blood. I am also cognizant of the importance of the study and would like to respond on behalf of my son/daughter.

I understand that I have the right to withdraw my son/daughter _____ from the research at any time, for any reason without penalty or harm. In case of withdrawal, I understand that the researchers will continue to take care of my son/daughter _____ like any other patient.

All the above conditions have been explained to me in the language, which I can understand. I agree that the samples could be sent abroad for further investigation.

Guardian's full name: _____Guardian's signature: _____ Date: _____

Child's full name: _____

Name of the physician: _____Signature:- _____

Witness: _____ Date: _____

Thank you for letting your child to participation in the study!

Thumbprint if subject is unable to sign

Appendix 8. Demographic characteristics, PCR-RFLP results for *Pfcrt* K76T and *Pfmdr1* N86Y codons and parasites/µl of *P. falciparum* samples analyzed

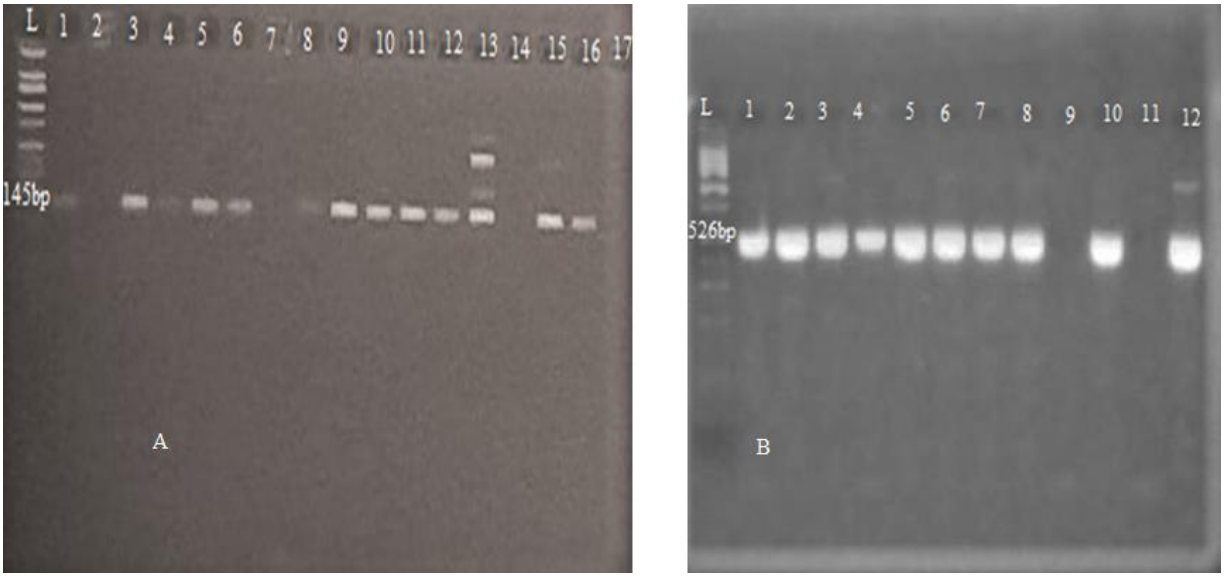
S. No.	**Sample code**	**Sex**	**Age**	**RFLP results of *Pfcrt* K76T**	**RFLP results of *Pfmdr1* N86Y**	**Parasites load/µl**
1.	MC-4006	M	58	76T	N86	4896
2.	An-4009	F	24	76T	"	6579
3.	Ad-4016	F	19	76T	"	1756
4.	MC-4056	F	28	K76	"	2086
5.	An-4002	F	20	76T	"	36143
6.	MC-4001	M	35	76T	"	480
7.	MC-4055	M	68	76T	"	4673
8.	G-6004	F	56	76T	"	417
9.	MC-4001	F	12	76T	"	27093
10.	MC-4063	F	20	76T	"	7136
11.	Ad-7015	F	28	76T	"	19843
12.	MC-4057	F	31	76T	"	43452
13.	MC-4054	M	42	76T	"	35624
14.	MC-4046	M	31	76T	"	585
15.	MC-4048	M	21	76T	"	19582
16.	An-4003	M	9	76T	"	22217
17.	MC-4045	F	30	76T	"	457
18.	MC-4003	F	37	76T	"	2040
19.	MC-4007	M	12	76T	"	6705

20.	BSH-103	M	15	76T	Not amplified	11586
21.	D-1007	M	38	76T	N86	6431
22.	MC-4015	M	46	76T	“	128
23.	BSH-105	M	3	76T	Not amplified	6274
24.	MC-4039	M	55	76T	N86	440
25.	G-4002	M	65	76T	“	2923
26.	W-5002	M	59	76T	“	1765
27.	W-5010	F	5	76T	“	950
28.	W-5035	F	17	76T	“	94
29.	W-5028	M	18	76T	“	11704
30.	W-5029	M	27	76T	“	4832
31.	W-5020	M	6	76T	“	1600
32.	W-5006	M	38	K76	“	5903
33.	W-5044	M	18	76T	“	24400
34.	W-5008	M	26	K76	“	6731
35.	DF-401	M	20	76T	“	5572
36.	W-5046	M	31	76T	“	20956
37.	W-5048	M	16	76T	“	33800
38.	W-5012	F	4	76T	“	6524
39.	W-5036	M	13	76T	“	3590
40.	W-5045	F	30	76T	“	1482
41.	W-5051	F	10	K76	“	4038
42.	W-5050	M	18	76T	“	46273
43.	W-5032	M	17	76T	“	79

44.	W-5007	M	12	76T	“	7255
45.	MT-0028	M	4	K76	“	2876
46.	MT-0027	M	20	K76	“	24827
47.	MT-0043	F	18	76T	“	18840
48.	MT-0025	M	3	76T	“	20640
49.	MT-0045	M	20	76T	“	12019
50.	MT-0048	F	30	76T	“	14217
51.	MT-0041	F	20	76T	“	62089
52.	MT-0026	M	15	76T	“	23720
53.	MT-0079	F	8	76T	“	9561
54.	MT-0090	M	10	76T	“	46698
55.	MT-0111	F	9	76T	“	6200
56.	MT-0089	M	6	76T	“	157538
57.	MT-0033	M	16	76T	“	20760
58.	MT-0017	M	30	76T	“	1564
59.	MT-0009	M	20	76T	“	330
60.	MT-0010	M	13	76T	“	803
61.	MT-0100	M	13	76T	“	25640
62.	MT-0105	M	26	76T	“	3960
63.	MT-0102	F	14	76T	“	8850
64.	MT-0117	M	12	76T	“	2318
65.	MT-0113	F	29	76T	“	22169
66.	MT-0109	M	36	76T	“	95
67.	MT-0112	M	12	76T	“	5076

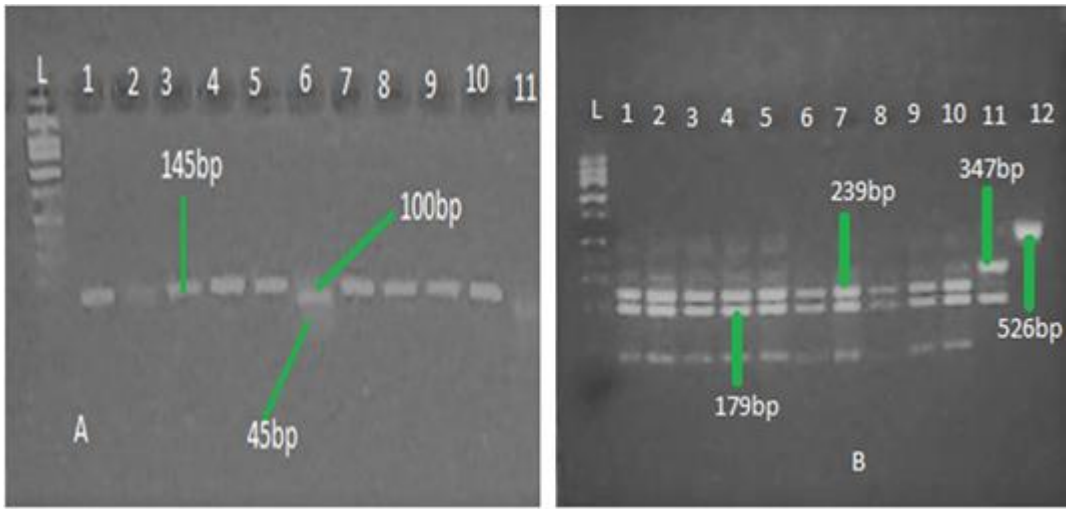
68.	MT-0098	F	15	76T	“	43586
69.	MT-0143	M	25	76T	“	836
70.	MT-0084	F	21	76T	“	474
71.	MT-0082	M	21	K76	“	6901
72.	MT-0118	F	38	76T	“	9137
73.	MT-0072	M	8	76T	“	148756
74.	MT-0062	M	1	76T	“	26156
75.	MT-0115	M	26	76T	“	245
76.	MT-0125	M	20	76T	“	16000
77.	MT-0002	F	23	76T	“	7219
78.	MT-0034	F	32	76T	“	1671
79.	MT-0076	M	28	76T	“	15778
80.	MT-0080	M	18	76T	“	2717
81.	MT-0066	M	31	76T	“	23369
82.	MT-0051	F	4	76T	“	4038
83.	MT-0116	M	45	76T	Not analyzed	7164

Appendix 9. Representative gel images showing nested 2 PCR products of *Pfcrt* K76T (A) and *Pfmdr1* N86Y (B) codons.



(**A**) L= 100bp DNA ladder marker, lanes 1-15 are samples, lane 16 is positive control (3D7) and lane 17 is negative control (water) (**B**) L= 100bp ladder marker, lanes 1-10 are samples, lane 11 is negative control (water), lane 12 is positive control (3D7). bp: base pair.

Appendix 10. Representative gel images showing digested nested 2 PCR products of *Pfcrt* K76T (A) and *Pfmdr1* N86Y (B) codons.



(A) K76T codon cleaved with *APoI* RE run in 2.0% agarose gel. L= 100bp DNA ladder marker, lanes 1-9 are samples, lane 10 is uncut control and lane 11 is wild-type control (*Pf*3D7). (**B**) N86Y codon cleaved with *APoI* RE run in 2.5% agarose gel. L= 100bp ladder, Lanes 1-9 are samples, lane 10 is wild-type pos. cont. (3D7), lane 11 is mutant-type pos. cont. (K1), and lane 12 uncut control. bp: base pair.

Appendix 11. Socio-demographic data, *Plasmodium* species and parasite counts used for membrane feeding assay

S.No.	**IDN**	**Sex**	**Age**	**Residents**	**Marital**	**SPP**	**Parasite level/µl**
1.	MC-4042	Female	60	Urban	Ever married	*P. vivax*	6667
2.	G-6002	Female	75	Urban	Ever married	*P. vivax*	587
3.	MC-4006	Male	58	Urban	Ever married	*P. falciparum*	506
4.	MC-4007	Male	12	Urban	Never married	*P. falciparum*	343
5.	W-5002	Male	59	Urban	Ever married	*P. falciparum*	1765
6.	W-5006	Male	38	Rural	Ever married	*P. falciparum*	5903
7.	MC-4015	Male	46	Rural	Ever married	*P. falciparum*	111
8.	AW0001	Male	52	Rural	Ever married	*P. falciparum*	1584
9.	DF-401	Male	20	Rural	Never married	*P. falciparum*	5572
10.	AW0002	Female	20	Rural	Ever married	*P. falciparum*	1428
11.	DF-402	Male	9	Rural	Never married	*P. falciparum*	15547
12.	A-1003	Female	30	Urban	Ever married	*P. falciparum*	1245
13.	W-5032	Female	17	Rural	Never married	*P. falciparum*	1786
14.	MC-4041	Female	22	Urban	Never married	*P. falciparum*	3060
15.	MC-4045	Female	30	Urban	Ever married	*P. falciparum*	1135
16.	MC-4054	Male	29	Urban	Never married	*P. falciparum*	780
17.	MC-4055	Male	68	Urban	Ever married	*P. falciparum*	142
18.	MC-4004	Male	25	Urban	Never married	*Mixed*	16302
19.	MC-4029	Male	40	Urban	Ever married	*Mixed*	5592
20.	MC-4028	Male	58	Urban	Ever married	*P. vivax*	4098
21.	MC-4032	Male	25	Urban	Ever married	*P. vivax*	8238

22.	W-5025	Female	75	Rural	Ever married	*P. vivax*	5627
23.	MT-0001	Male	22	Rural	Ever married	*P. vivax*	8328
24.	MC-4031	Male	45	Urban	Ever married	*P.vivax*	42833
25.	MC-4043	Male	22	Urban	Never married	*P. vivax*	7843
26.	W-5040	Male	19	Rural	Never married	*P. vivax*	6634
27.	MC-4053	Male	22	Urban	Never married	*P. vivax*	6728
28.	W-5003	Female	26	Rural	Never married	*P. vivax*	1308
29.	MC-4023	Male	30	Urban	Ever married	*P. vivax*	1588
30.	MC-4036	Male	45	Urban	Ever married	*P. vivax*	634
31.	MC-4049	Female	30	Urban	Ever married	*P. vivax* .	6920
32.	MC-4046	Male	31	Urban	Never married	*P. vivax*	1233
33.	MC-4026	Male	30	Urban	Ever married	*P. vivax*	9240
34.	MC-4008	Male	40	Urban	Ever married	*P. vivax*	1223
35.	MC-4019	Female	21	Urban	Never married	*P. vivax*	5791
36.	MC-4025	Male	20	Urban	Never married	*P. vivax*	14886
37.	W-5024	Female	38	Rural	Ever married	*P. vivax*	2371
38.	MC-4030	Male	48	Urban	Ever married	*P. vivax*	3626
39.	MC-4033	Female	20	Urban	Ever married	*P. vivax*	14410
40.	MC-4034	Male	57	Urban	Ever married	*P. vivax*	4640
41.	W-5031	Male	17	Rural	Never married	*P. vivax*	751
42.	MC-4037	Male	18	Urban	Never married	*P. vivax*	8280
43.	MJ-7001	Female	25	Rural	Ever married	*P. vivax*	5970
44.	AW-0007	Male	29	Rural	Never married	*P. vivax*	3677
45.	H-9003	Male	26	Urban	Never married	*P. vivax*	789

46.	MC-4050	Male	39	Urban	Ever married	*P. vivax*	6338
47.	MC-4052	Male	22	Urban	Never married	*P. vivax*	6037
48.	MC-4005	Male	22	Urban	Never married	*P. vivax*	8281
49.	MC-4016	Male	25	Rural	Never married	*P. vivax*	13678
50.	A-1002	Female	34	Urban	Ever married	*P. vivax*	1618
51.	MC-4014	Male	20	Urban	Never married	*P. vivax*	419
52.	MC-4021	Male	28	Urban	Ever married	*P. vivax*	7203
53.	MC-4022	Male	28	Urban	Never married	*P. vivax*	11902
54.	MC-4024	Male	19	Urban	Never married	*P. vivax*	10000
55.	W-5016	Female	8	Rural	Never married	*P. vivax*	914
56.	W-5017	Male	75	Rural	Ever married	*P. vivax*	2615
57.	WH-5034	Male	31	Rural	Never married	*P. vivax*	1592
58.	WH5039	Female	34	Rural	Ever married	*P. vivax*	879
59.	MC-4048	Male	21	Urban	Never married	*Mixed*	19582
60.	MC-4011	Female	55	Urban	Ever married	*P. vivax*	3766
61.	MC-4018	Male	21	Urban	Never married	*P. vivax*	7208
62.	MC-4038	Male	48	Urban	Ever married	*P. vivax*	8080
63.	WH-5033	Female	30	Rural	Ever married	*P. vivax*	14582

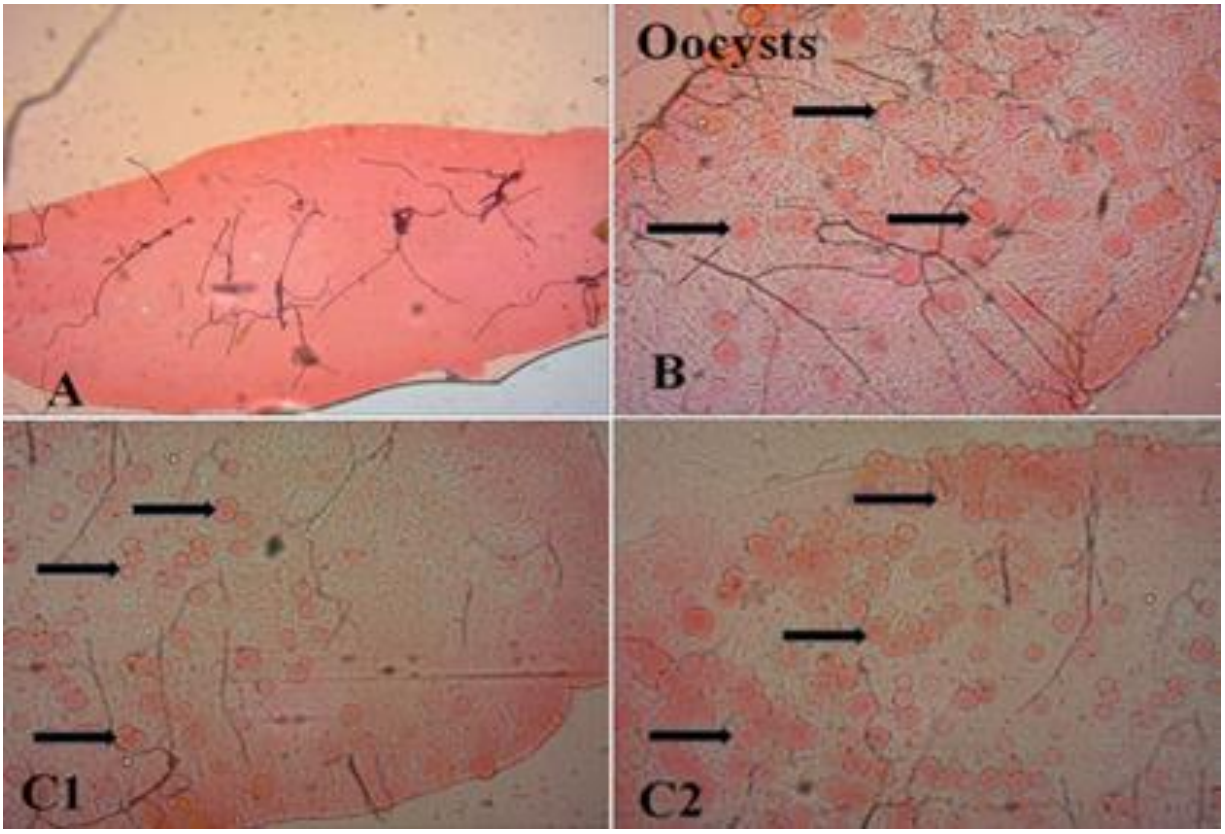
Appendix 12. Data of mosquito infectivity by membrane feeding assay

S. No.	Gametocyte level/µl	number of exposed mosquitoes	number of fully fed mosquitoes	Number of mid-gut dissected mosquitoes(7dpi)	Number of oocysts infected mosquitoes
1.	71	120	30	26	1
2.	0	120	25	10	0
3.	0	200	70	8	0
4.	0	160	111	30	0
5.	0	50	18	10	0
6.	0	160	153	20	0
7.	48	160	132	46	1
8.	56	120	108	32	6
9.	0	90	51	6	0
10.	61	120	89	40	15
11.	0	140	101	20	0
12.	0	120	75	35	1
13.	81	160	142	50	3
14.	74	240	74	30	2
15.	0	140	63	39	1
16.	0	160	100	50	0
17.	0	120	82	15	0
18.	98	160	31	11	0
19.	50	160	83	11	2
20.	78	160	116	5	1
21.	64	90	65	16	2

22.	128	120	82	14	8
23.	151	550	324	12	4
24.	46	90	70	8	0
25.	9	80	33	11	1
26.	117	160	123	11	6
27.	4	160	114	30	12
28.	388	200	73	50	2
29.	157	240	127	13	6
30.	0	120	91	18	0
31.	8	90	46	33	20
32.	0	160	58	35	2
33.	80	40	26	9	1
34.	165	280	206	60	24
35.	225	240	195	26	0
36.	4	200	148	7	4
37.	97	160	121	8	3
38.	5	120	91	9	2
39.	2395	90	65	26	26
40.	80	120	80	15	0
41.	0	100	81	8	6
42.	360	120	93	7	5
43.	563	330	180	11	8
44.	258	320	206	10	9
45.	0	120	69	33	0

46.	172	480	330	18	15
47.	7	160	120	32	5
48.	275	160	38	6	0
49.	2320	240	186	16	14
50.	67	240	153	21	14
51.	0	160	21	10	0
52.	199	240	164	24	7
53.	78	200	113	28	13
54.	180	240	119	20	7
55.	2	160	89	21	2
56.	52	240	123	6	1
57.	65	160	132	31	4
58.	43	320	193	60	6
59.	0	120	82	50	0
60.	679	120	84	10	5
61.	102	160	126	21	0
62.	80	240	143	22	0
63.	2785	200	158	28	25

Appendix 13. Typical images of mid-gut dissected from mosquitoes fed on *P. vivax* infected blood.



Mid-gut oocyst negative from one mosquito (**A**), mid-gut oocyst positive from one mosquito (**B**), two images of oocyst positive mid-gut from one mosquito (**C1** and **C2**). Arrows point towards the oocyst. The image was viewed at 20x magnification and taken with Pylon Viewer Camera Software installed on a desktop.

Appendix 14. Data of mosquito infectiousness

S. No.	Number of oocyst per assay	Number of salivary gland dissected mosquitoes (14dpi)	Number of sporozoites infected mosquitoes	Number of sporozoites per assay	Log10 parasite level/ µl
1.	5	10	0		3.82
2.	0				2.77
3.	0	8	0		3.70
4.	0				3.80
5.	0				3.25
6.	0				3.77
7.	5	10	0		2.05
8.	48	7	0		3.20
9.	0				3.75
10.	24	8	0		3.15
11.	0				4.19
12.	4				3.10
13.	127				3.25
14.	7				3.49
15.	11				3.05
16.	0				2.89
17.	0				2.15
18.	0				4.21
19.	12	21	1	81	3.75
20.	9	12	1	75	3.61

21.	9				3.92
22.	46	2	0		3.75
23.	136				3.92
24.	0	11	0		4.63
25.	9				3.89
26.	192	14	3	1015	3.82
27.	232				3.83
28.	6	3	0		4.29
29.	156	3	1	87	3.20
30.	0				2.8
31.	1469				3.84
32.	8				3.09
33.	8	4	0		3.97
34.	588	11	5	152	3.09
35.	0				3.76
36.	41	8	1	79	4.17
37.	23	4	0		3.37
38.	6	7	0		3.56
39.	2415	8	1	367	4.16
40.	0				3.67
41.	42	7	0		2.88
42.	126	5	1	86	3.92
43.	55	15	3	295	3.78
44.	601	22	13	1337	3.57

45.	0				3.90
46.	538				3.80
47.	9				3.78
48.	0				3.92
49.	1650	13	9	567	4.14
50.	66	2	0		2.79
51.	0				2.62
52.	78	1	1	56	3.86
53.	82	5	1	63	4.08
54.	334	9	4	232	4.00
55.	6	14	0		2.96
56.	24	2	1	92	3.42
57.	8				4.20
58.	11				2.94
59.	0				4.29
60.	83	16	5	301	3.58
61.	0				3.86
62.	0				3.91
63.	2625	22	7	548	4.15

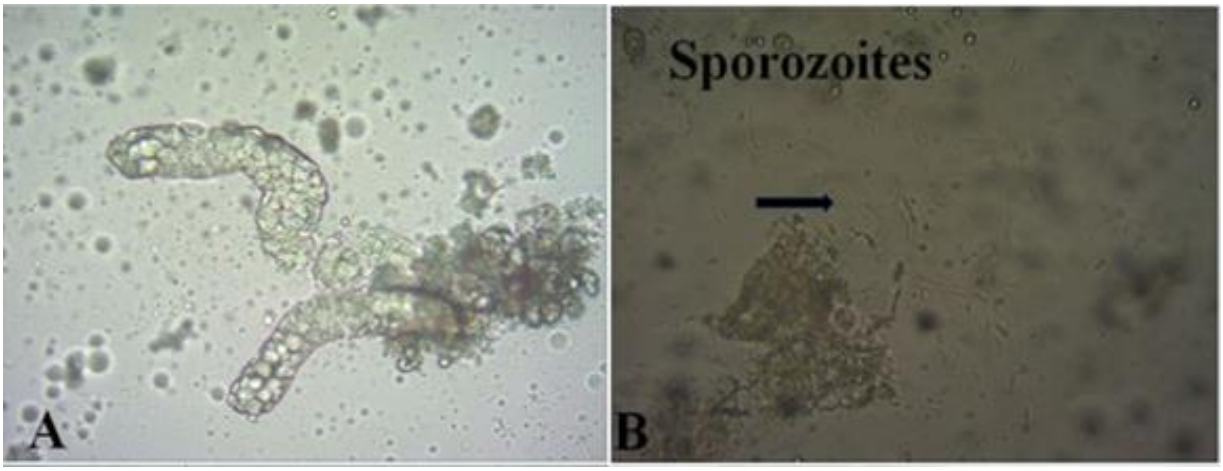
Appendix 15. Log10 converted oocysts and gametocytes

S.No.	Log10 Oocyts per assay	Log10 gametocyte level/µl
1.	0.70	1.85
2.		
3.		
4.		
5.		
6.		
7.	0.70	1.68
8.	1.70	1.75
9.		
10.	1.38	1.79
11.		
12.	0.60	
13.	2.10	1.91
14.	0.85	1.87
15.	1.04	
16.		
17.		
18.		1.99
19.	1.08	1.70
20.	0.95	1.89
21.	0.95	1.81
22.	1.66	2.11

23.	2.13	2.18
24.		1.66
25.	0.95	
26.	2.28	2.07
27.	2.37	
28.	0.78	2.59
29.	2.19	2.20
30.		
31.	3.17	
32.	0.90	
33.	0.90	1.90
34.	2.77	2.22
35.		2.35
36.	1.61	
37.	1.36	1.99
38.	0.78	
39.	3.38	3.38
40.		1.90
41.	1.62	
42.	2.10	2.56
43.	1.74	2.75
44.	2.78	2.41
45.		
46.	2.73	2.24

47.	0.95	
48.		2.44
49.	3.22	3.37
50.	1.82	1.83
51.		
52.	1.89	2.30
53.	1.91	1.89
54.	2.52	2.26
55.	0.78	
56.	1.38	1.72
57.	0.90	1.81
58.	1.04	
59.		
60.	1.92	2.83
61.		2.01
62.		1.90
63.	3.42	3.44

Appendix 16. Typical images of salivary glands dissected from mosquitoes fed on P. vivax infected patients.



Sporozoite-negative mosquito (A), sporozoite-positive mosquito (B). Arrow points towards the sporozoites released from the salivary glands. The image was viewed at 40x magnification and taken with Pylon Viewer Camera Software installed on a desktop.