Therapeutic Efficacy of Artemether Lumefantrine with (s) Primaquine for the treatment of uncomplicated *Plasmodium falciparum* in Bambasi District, Benishangul-Gumuz, Ethiopia

Jimma Dinsa

A Thesis Submitted to the Department of Applied Biology, School of Applied Natural Science Presented in Partial Fulfillment of the Requirment for the Degree Master's of Science in Biology, Specialization in Biotechnology

Office of Graduate Studies
Adama Science and Technology University

March 2022
Adama, Ethiopia

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DECLARATION

I hereby declare that this Master's Thesis entitled **"Therapeutic Efficacy of Artemether Lumefantrine with (s) Primaquine for the treatment of uncomplicated *Plasmodium falciparum* in Bambasi District, Benishangul-Gumuz, Ethiopia"**is my original work. Thus, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation

Name: Jimma Dinsa **Signature** **Date:**

RECOMMENDATION

We, the advisors of this thesis, hereby certify that, we have read the revised version of the thesis entitled **"**Therapeutic Efficacy of Artemether Lumefantrine with (s) Primaquine for the treatment of uncomplicated *Plasmodium falciparum* in Bambasi District, Benishangul-Gumuz, Ethiopia**"** prepared under our guidance by **Jimma Dinsa** submitted in partial fulfillment of the requirements for the degree of Mater's of Science in Biotechnology. Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

Bayissa Chala (PhD)

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APPROVAL SHEET

I/we, the advisors of the thesis entitled **"Therapeutic Efficacy of Artemether Lumefantrine with (s) Primaquine for the treatment of uncomplicated *Plasmodium falciparum* in Bambasi District, Benishangul-Gumuz, Ethiopia"** and developed by **Jimma Dinsa**, hereby certify that, the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor: **Bayissa Chala (PhD)**	Signature	Date
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We, the undersigned, members of the Board of Examiners of the thesis by **Jimma Dinsa** have read and evaluated the thesis entitled **"Therapeutic Efficacy Artemether Lumefantrine with (s) Primaquine for the treatment of *Plasmodium falciparum* in Bambasi District, Benishangul-Gumuz, Ethiopia"** and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Biotechnology.

Chairperson	Signature	Date
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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

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ACKNOWLEDGMENTS

First and foremost, I give honor to the Almighty God for every protection he did for me. I would like to express my deepest gratitude to my advisors Dr. Bayissa Chala and Dr. Endalamaw Gadisa for their persistent mentorship and excellent guidance, reading the thesis and forwarding constructive comments throughout my thesis write up. In addition, my thanks go to FMoH Malaria control and prevention Program for the drug donation. Finally, also AHRI for the financial support to carryout my thesis sample collection and laboratory analysis consumables.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACPR	Adequate clinical and parasitological response
AL	Artemether-Lumefantrine
CQ	Chloroquine Phosphate
CRFs	Case report forms
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ETF	Early treatment failure
G6PD	Glucose 6 phosphate deficiency
HRPII	Histidine rich protein-II
IRBCs	Infected Red blood cells
LAMP	Loop-mediated isothermal amplification
LCF	Late clinical failure
LPF	Late parasitological failure
MDA	Mass drug administration
MSP1	Merozoite surface protein-1
MSP2	Merozoite surface protein-2
PCR	Polymerase chain reaction
PLDH	Lactate dehydrogenase
Pfmsp2	*Plasmodium falciparum* merozoite surface protein-2
RDTs	Rapid diagnostic tests
SD-PQ	Single dose Primaquine
SSA	Sub-Saharan Africa
TES	Therapeutic efficacy studies
TPP	Total patients per-protocol
WHO	World Health Organization

ABSTRACT

The therapeutic efficacy and safety of standard artemether-lumefantrine plus single dose primaquine for the treatment of uncomplicated Plasmodium falciparum is not yet assessed and poorly documented in Bambasi, North Western Ethiopia. As per the recommendation of World Health Organization every two year, regular monitoring of antimalarial drug efficacy is an important tools of supporting national malaria treatment policy. The study was investigated the efficacy and safety of AL plus (s) PQ treatment for P. falciparum malaria in Ethiopia. One arm longitudinal prospective, evaluated clinical, safety and parasitological response, directly observed treatment during 42 days of follow up visits with AL plus (s) PQ for 6 months and older ages of participants with uncomplicated P. falciparum malaria in Bambasi district, Ethiopia. A study was based on an updated protocol of World Health Organization 2009 recommendations for the surveillance of anti-malarial drug efficacy with primary outcomes of clinical and parasitological cure rate were at day-42. The secondary outcomes, safety, fever, asexual parasite and gametocyte clearance were assessed. The cure rate at day-42 of microscopy and PCR were compared using per-protocol (PP) analysis. Nested polymerase chain reaction (nPCR) was used for confirmatory test. While msp2-nPCR used for PCR-correction and P. falciparum parasite genotyping of baseline and at the time of recurrences to distinguish between true treatment failure (recrudescence) and new infections. Of the 123 enrolled study participants, 52 completed day-42 with ACPR, 14 loss to follow up and 57 individuals with parasite reoccurrences onwards from day-14 with falciparum, mixed and P. vivax infections. A pair of 41 samples of falciparum infections were msp2-PCR-genotyped and 14.3% treatment failure (12 recrudescence) was observed using WHO Kaplan-Meier survival estimation. The per-protocol (PP) cure rate of AL plus (s) PQ of PCR-uncorrected and PCR-correction were 55.2% (95% CI 44.8-64.4) and 85.7% (75.8-91.7%) respectively. While 22.5% (12) of the parasite recurrences were P.vivax co-infections contribute for microscopic treatment failure. In this finding, delayed parasite clearance rate was observed whereas gametocyte was exponentially decreased and cleared after day-3 visiting. The delayed asexual parasite clearance is a good indicator of either partial or multi-drug resistance. A large scale molecular surveillance to assess the possible mechanisms of artemisinin-tolerate parasites in the area is advised to provide more information.

Keywords: Artemether-lumefantrine, Efficacy, Primaquine, s PQ, MOI, msp2, Bambasi.

1. INTRODUCTION

1.1 Background of the Study

Malaria due to *Plasmodium falciparum* is the most common devastating human infection that accounts about 99% in sub-Saharan African (SSA) countries (WHO, 2019). In WHO African region in 2018, also accounted 99.7 % of malaria cases with comparatively rarely occurring *P. vivax* co-infection in respective region (Padron *et al.,* 2021). It's one of the leading causes of morbidity and mortality globally, particularly in the sub-Saharan countries and also it is a major obstacle to socio-economic development in the country (Larson, 2019).

The World malaria report of 2020 estimates that, there were 229 million malaria cases worldwide in 2019, with total estimated death of 409,000 (Al-Awadhi *et al.*, 2021). According to the report, the most affected group were children under five, accounting for about 67 % (274,000) deaths worldwide. Global malaria burden showed a decline in 2000; around 238 million and 736,000 new cases and death rates were observed, respectively. Nearly 80% of global malaria deaths in 2017 were concentrated in 17 countries in the WHO African Region (WHO, 2020). The sub-Saharan Africa region was the most affected area contributing a higher share of malaria cases and deaths.Malaria cases and mortality rates have been reduced globally in the past two decades. Following this, the incidence rate per 1000 population was reduced from 80 in 2000 to 57 in 2019 and similarly,mortality rate was reduced from 25 in 2000 to 10 in 2019. Ethiopia is one of the malarious countries in the sub-Saharan region (Kendie *et al.*, 2021), with the prevalence of *P. falciparum* (60%) and *P. vivax* (40%) cases, respectively (Alemu *et al.,* 2011). Despite the high risk of malaria transmission, facility-based evaluation of antimalarial drug efficacy must be done as per the WHO recommendations that help in early drug resistance management and mistreatment (Girum *et al.,* 2019).

The transmission of malaria is unstable and influenced by several environmental factors such as topography, rainfall, climate, and socio-economic conditions. For this reason, tropical regions, including Ethiopia with warm temperature, heavy rainfall and high humidity are conducive for mosquito breeding, longevity and parasite sporogony (Feleke *et al.*, 2018). In Ethiopia, malaria transmission bi-peaks annually related to the main rain season from September to December and short rain season from April to May and areas located less than 2,000 meters above the sea level are consideredas malarious (Deress and Girma, 2019). Even though the burden of malaria was reduced remarkably through public health intervention

measures, it is still a major public health problem in Ethiopia. About 68% of the population lives in malarious region that needs clinical and good laboratory based treatment for well drug administration (Taffese *et al.*, 2018). In Benishangul Gumuz regional state, almost all districts (98%) of the landmass are malarious area and 97% of the population are at risk for malaria infection (Gontie *et al.*, 2020). The annual malaria prevalence in the region is 265 per population of 1000 cases, while the national malaria prevalence cases were 37 per 1000 populations stated by FMOH 2015/16 (Kirigia and Wambebe, 2006). The prevalence of *P. falciparum* and *vivax* infections is about 91% and 9% respectively in Menge districts found in the same zone of the study site with decreased death rate of 8 to 4 in the past few years reported (Mohammed and Animut, 2019). However, this rate is still greater than the plan of FMOH, which is expected to be near zero death of malaria at end of 2020 years in Ethiopia due to serious of measures of intervention and integrated vector control (Mohammed and Animut, 2013).

Globally, artemisinin based combination therapy (ACT) is recommended by WHO as a first line treatment for uncomplicated *P. falciparum*. Artemisinin resistance in *P. falciparum* has been reported in Southeast Asia with a decreased rate of treatment failures similar in some African counties (Yeshiwondim *et al.*, 2010). The treatment failures rate more than 10% leads to change the national antimalarial treatment policy per the WHO treatment guide line for both arm (WHO, 2009). Antimalarial drug resistance is one of the major obstacles for malaria control and curtails the life span of several drugs. Following a widespread failure of chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) to both *P. vivax* and *P. falciparum,* respectively, most of the malaria-endemic countries had shifted their malaria treatment policy to artemisinin-based combination therapy (ACT) (Deressa *et al.*, 2017).

Several studies assessed on the efficacy of antimalarial agents in Ethiopia and reduced efficacy of CQ has forced a change in the selection of antimalarial drug in the management of *P. falciparum* malaria (Nega *et al.*, 2016). Chloroquine resistance to *P. falciparum* studies conducted between 1997 and 1998, demonstrated a total treatment failure rate of 65% on 18 sites (FMoH, 1998). Due to this, Ethiopia has also adopted artemether-lumefantrine (AL) and CQ as first-line treatment for *P. falciparum* and *P. vivax* respectively since 2004 (Hwang *et al.*, 2011). At the time of use, its efficacy was about 99.1 % with a treatment failure rate of below 1% which is almost negligible. Overall, the WHO malaria control programs recommends

efficacy above 90% clinical and parasitological curative rates of any antimalarial drugs to be approved as first line treatment.

Scaling-up of control interventions and improved antimalarial chemotherapy is very important in reduction of malaria prevalence. One of the key strategies set by WHO for pre-elimination is the addition of single-low dose (0.25mg/Kg) of primaquine for uncomplicated *P. falciparum* in low endemic settings. It's the only anti-malarial drug currently approved for radical cure of relapsing *P.vivax* and *Ovale* in addition to mature gametocyte clearance of *P.falciparum (WHO, 2015)*. Most of the previously reported data reveals that, the single low-dose of primaquine (SLD-PQ) to be highly efficient against the naturally transmissible parasite stage of mature *P. falciparum* gametocyte that helps in reducing gametocyte circulation between human-mosquito (Lin *et al.,* 2017). The asexual parasite and early stage gametocyte and partial mature gametocyte rapidly clears and allowing partial persistent of gametocyte which remain infectious after treatment that would be sustaining for transmission cycle (Bousema *et al.*, 2006).

One of the major concerns following the addition of single-low dose of PQ to standard AL for *P. falciparum* in low transmission settings or where resistant to artemisinin threatened as a key component for pre-elimination is the safety issues of the individuals (patients)(Von Seidlein *et al.*, 2013). The safety of the patients is correlated with haemolytic, particularly related to the glucose six dehydrogenase enzyme deficient (G6PDd) individuals. In most of this X-linked deficiency, the red blood cells susceptibility increases oxidative stresses and thus haemolysis which is prevalent in most of the tropical countries. Some of the major factors among the indicators of severity of haemolysis were doses of PQ, gender, duration of exposure and presence of variant G6PDd (Hill *et al.,* 2006).

Several studies conducted related to the safety and tolerability of the SD-PQ (0.25mg/Kg) in some African countries like Tanzania, Senegal and South Africa and finally they recommended the deployment of PQ for their pre-elimination strategies in low transmission areas for uncomplicated *P. falciparum* (Poirot *et al.*, 2016). In Senegal, no patients had clinically important reduction in Hgb concentrations. And also in Thailand, repeated doses of 0.25 mg/kg of primaquine in addition to DHA-PQ during mass drug admistration campaigns in healthy adults not show clinically significant reduction in Hgb concentration (Tine *et al.*, 2017). Primaquine application also substantially reduces the gametocyte carriage in G6PD normal patients reported in Mali (Dicko *et al.,* 2016) and Burkina Faso. Therefore, the only antimalarial

treatment that specifically target gametocide is 8-aminoquinoline primaquine in both *P. falciparum* and *P. vivax* (Amaratunga *et al.*, 2014).

Currently, FMOH also recommends the single dose (0.25 mg/Kg (BW) of PQ with standard AL for uncomplicated *P. falciparum* treatment in low transmission settings in our country (Argaw *et al.*, 2019). The application of PQ is very important for the clearance of mature gametocyte and blood infections preventing treatment failure and relapses for malaria elimination. The first G6PD screening was conducted across Southwestern districts of Ethiopia (Lo *et al.*, 2019). howver, the lack of level of information and national status of G6PD based on geographical location, age and sex were not well documented and the uncertainity of safety of single dose and long term PQ exposure (14 days) poses risk to malaria patients. Additionally, the investigation of safety, tolerability and efficacy of (s) PQ in addition to artemisinin remain limited to certain malaria endemic countries. However, no evidence based investigation was yet assessed in Ethiopia on the safety and efficacy of (s) PQ plus AL in an uncomplicated *P. falciparum* treatment. Therefore, this study was was aimed at assessing the safety, tolerability and efficacy of this combination therapy regardless of G6PDd test prior to its deployment. Most of the previously reported data related to PQ application were excluded breast feeding women, infant children, pregnant women, acute heamoglobin concentration and sever malaria cases from the study due the haemolytic effects of primaquine with artemisinin combination therapy (Mwaiswelo *et al.*, 2016b). However, it's difficult to halt the onward transmission cycle of malaria parasite between human to mosquito and vice versa excluding those individuals. A few years onwards, some of the malaria endemic countries were exercising the application of PQ in their health policy as a pre-elimination strategies to halt the gametocyte circulation. Adding of SD-PQ with AL conducted in Tanzania in randomized clinical trials in uncomplicated *P.falciparum* did not add value to the efficacy of AL without PQ in comparison (Mwaiswelo *et al.*, 2016a). In both arms of study, in crude and PCR-corrected, reinfection were confirmed, while the asexual parasite was rapidly cleared in both arms and overall, with efficacy of in line with WHO threshold.

One of the key point to be considered is how to use primaquine as a pre-elimination strategies. The two strategies are; one is adding PQ in treating the symptomatic patients by identifying and treating all as mass screen cases (MSaT) and the other is the mass drug administration (MDA) programs as a key component which needs more drugs even for not ill in this section. Treating only symptomatic cases (small fraction) may not bring the substantial reduction of

malaria transmission and MDA is a key strategies for pre-elimination regardless of huge drug consumption and may be the enforcement of taking drugs without malaria infections. Therefore, this longitudinal prospective study was conducted in Bambasi district, Benishangul-Gumuz, Ethiopia, during the malaria transmission season. In this study, microscopically confirmed symptomatic cases of uncomplicated *P. falciparum* mono-infections at health center were enrolled per the WHO standardized efficacy protocol. The primary outcome of this study was to monitor the safety and efficacy of AL plus (s) PQ for *P. falciparum* as a routine follow up study in patients age greater than 6 months and produce baseline data for the malaria control and prevention program.

1.2 Statement of the Problem

Apart from the lack of commitments to implement preventive strategies and lack of funds in malaria-endemic countries, the emergence of parasite resistance to the currently working antimalarial medicines and of mosquito resistance to insecticides threatens the malaria elimination campaign globally (WHO, 2015a). For example, according to the artemisinin resistance tracking collaboration report, the kelch13 mutation associated with ACT resistance has been reported in Southeast Asia since 2007. As a result, researchers called an urgent action to eliminate *P.falciparum* malaria, mainly in the Greater Mekong Sub region, before spreading and expanding to other malaria regions (Hamilton *et al.,* 2019). Similarly, in east Africa like Uganda, the Falciparum artimesinin resistance was reported (Nass and Efferth, 2019).

Antimalarial drug efficacy evaluation is the critical issues as per the WHO recommendation especially in malaria endemic areas by ensuring no one is mistreated due to in appropriate speciation during the first drug initiation and post treatment follow up diagnosis. In malaria endemicity areas, multiplicity of infection is common and needs attention to manage the cases with the course of first line treatment. In the management of malaria multiple infection (mixed), the FMOH national treatment guide line cluster under *P. falciparum* treatment monotherapy algorism previously. Hower, the first line treatment (ACT) alone can only rapidly clears asexual parasite, early stage gametocyte and partial mature gametocyte. This allowing partial persistent of gametocyte which remain infectious after treatment that would be sustaining for transmission cycle. The treatment faluire exceeding more than 10% forced to change the treatment policy. However, it's very important to ensure the applied treatment was implemented appropriately as moderate or high transmission settings stated previously on WHO updated treatment guide line before descision.

Recently, primaquine is the only approved gametocytocidal drug given as a single dose (0.25mg/kg) with the first line ACT treatment (WHO, 2015c). The ACT alone cannot clear mature *P. falciparum* gametocyte rather than active against immature gametocyte and asexual parasite and thus, especially challenging in malaria co-endemic settings. Currently, ministry of health, Ethiopia, recommends (s) PQ as a pre-elimination strategies that is under ongoing evalution prior to deployments. However, the application of PQ for the radical cure and at full dosage can cause acute haemolysis without G6PDd test. Thse may occur especially in low hemoglobin concentration patients, infant child and for both pregnant and breast feeding women for the safety issues. Furthere more, the efficacy and safety assessment of the study drug was not yet documented at Bambasi district and other similar malarious sentinel site in Ethiopia. Therefore, the aim of this study was to assess the safety, tolerablity and efficacy of AL + (s) PQ in uncomplicated *P. falciparum* and to compare the microscopy end points with the PCR-correction.

1.3 Objectives

1.3.1 General Objective

- To assess the therapeutic efficacy and safety of standard artemether-lumefantrine plus a single dose primaquine for the treatment of uncomplicated *P. falciparum* co-endemic settings in Bambasi, Benishangul-Gumuz, Ethiopia

1.3.2 Specific objectives

- To assess the clinical and parasitological efficacy of artemether-lumefantrine + single dose PQ in uncomplicated *P. falciparum* malaria patients above 6 months of age
- To compare the efficacy of microscopically treated artemether-lumefantrine plus PQ in *P. falciparum* with PCR-correction
- To quantify the level of misdiagnosed and mistreated cases at baseline under co-endemic settings
- To assess safety and adverse effect of AL+ PQ in uncomplicated *P.falciparum* treated patients
- To distinguish recrudescence from new infection by genotyping merozoites surface protein-2 genetic marker

1.4 Significance of the Study

As artimesinin drug resistance intensified, especially in east Africa like Uganda, concern began to grow that treatment policies would need to eventually change in favor of more efficacious drugs. Therefore, the present studies, significant to develop the evidence based policy for treatment of malaria, as the results of in vivo tests conducted using WHO standardized protocol. Additionally, to provided valuable information on the efficacy of antimalarial drugs in current use as well as initial evaluations of potential alternative treatment regimens (Nass and Efferth, 2019). Produce also a baseline data for the malaria control and prevention program. Moreover, it is important as an input data for national malaria treatment guide line.

1.5 Operational Definition

Early Treatment Failure (ETF): Danger signs or severe malaria on days (D 1, D 2 or D 3) in the presence of parasitemia and parasitemia on day 2 higher than on D 0, irrespective of axillary temperature, parasitemia on day 3 with axillary temperature ≥37.5 ºC and parasitemia on day 3 ≥25% of count on day 0

Late clinical failure (LCF): Danger signs or severe malaria with parasites any day from 7 to 42, or parasites any day from day 7 to 42 with fever or history of fever

Late parasitological failure (LPF): Presence of parasitemia on any day between day 7 and day 42 and axillary temperature <37.5 ºC in patients who did not previously meet any of the criteria of Early Treatment Failure or Late Clinical Failure

Adequate Clinical and Parasitological Response (ACPR): Absence of parasitemia on day 42 irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure, or late parasitological failure

Recrudescence: Recurrence (between day 7 to 42 following antimalarial treatment) of asexual parasitemia that is shown by PCR to comprise the same genotype/s that caused the original illness. **Recurrence**: Reappearance of asexual parasitemia after treatment, due to recrudescence, relapse (in *P. vivax* and *P. ovale* infections only) or a new infection

Reinfection: A new infection that follows a primary infection; can be distinguished from recrudescence by the parasite genotype, which is often (but not always) different from that which caused the initial infection

Relapse: Recurrence of asexual parasitemia in *P. vivax* or *P. ovale* infections arising from hypnozoites. Note: Relapse occurs when the blood-stage infection has been eliminated but hypnozoites persist in the liver and mature to form hepatic schizonts and backs to bloodstream

2. LITERATURE REVIEW

2.1Biology of Malaria Parasite

The history of malaria involves the correlation between the cyclical infections of malaria parasite in human and female Anopheles mosquitoes. This parasite grows and multiplies in the liver cells by destroying red blood cells and this occurs after the female Anopheline mosquito feed infected human blood. The blood-stage of malaria parasite cause different symptoms, which occurs both in male and female gametocytes. Then, they are ingested during a blood meal. They mate in the mosquito's gut, in which they begins cycles of growth and multiply. After 10-18 days, the forms of a parasite called sporozoites migrates to the mosquito's salivary glands, and thus, the infected mosquito carries disease (acting as a ''vector'') as stated below in Fig. 1.

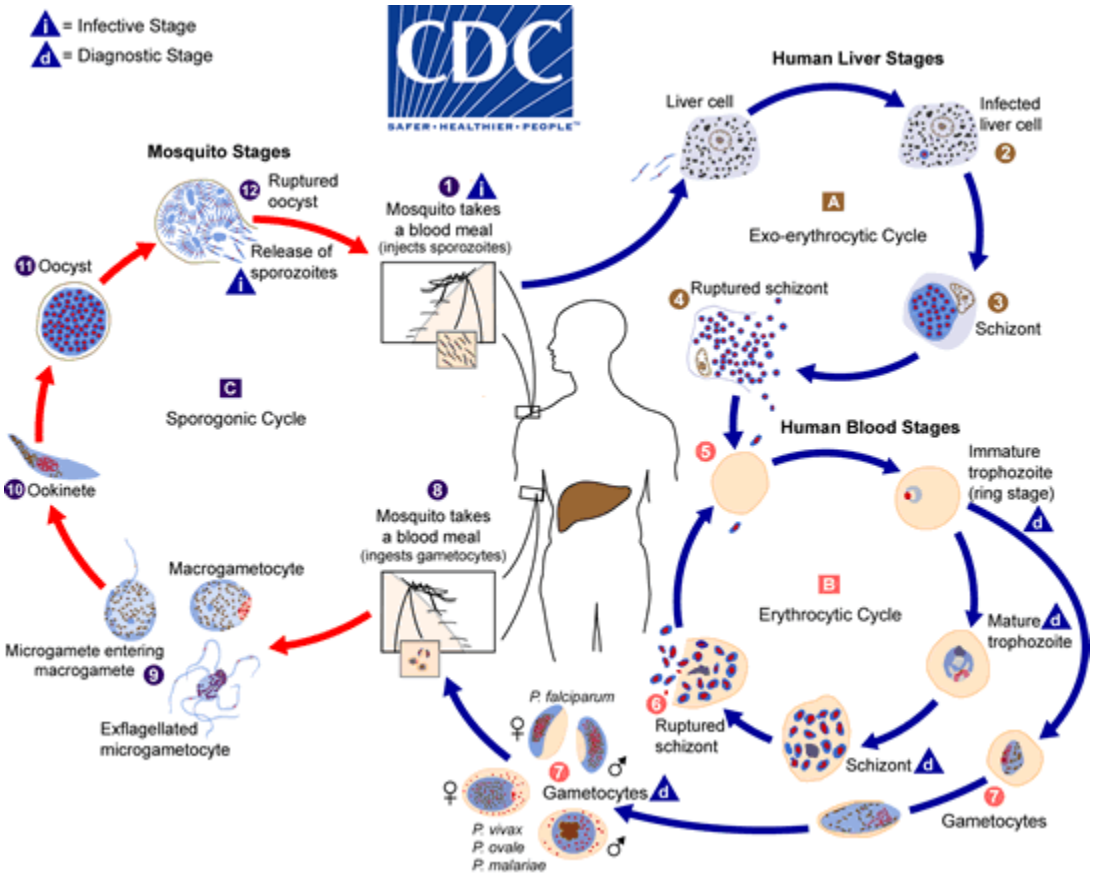


Figure 1: Life cycle of malaria parasite
Sources:http://www.samaritanid.com/MalariaCycle.html

2.1.1 Parasites

Malaria is the most devastating parasitic disease of humans, caused by the unicellular protozoa of the genus *Plasmodium*. *Plasmodium*, a single-celled parasite infects humans and cause illness (Dekel *et al.*, 2017). These are; *P. falciparum, P. malariae, P. vivax, P. ovale* and *P. knowlesi*. *Falciparum malaria* is potentially life-threatening human infections. Patients with severe *falciparum* malaria may develop liver and kidney failure, convulsions, and coma (Warrell, 2017). Although occasionally, severe infections with *P. vivax* and *P. ovale* generally cause less serious illness, but the parasites can remain dormant in the liver for many months, causing a reappearance of symptoms months or even years later. *P. falciparum* is responsible for the majority of malaria deaths globally and is the most prevalent species in sub-Saharan Africa (Takem *et al.*, 2014). The remaining species are not typically as life threatening as *P. falciparum*. *P. vivax*, is the second most significant species and is prevalent in Southeast Asia and Latin America (Carlton *et al.*, 2013). *P. vivax* and *ovale* have the added complication of a dormant liver stage, which can be reactivated in the absence of a mosquito bite, leading to clinical symptoms. *P. ovale* and *malariae* represent only a small percentage of infections. A fifth species *P. knowlesi*; a species that infects primates- has led to human malaria, but the exact mode of transmission remains unclear (Demogines *et al.*, 2012).

2.1.2 Vectors

In a history of vectors, a few century ago, researchers discovered certain species of insects that were responsible for the transmission of some diseases. In order to transmit the diseases, a disease carrier called ‘’vector’’ needs to live enough for the parasite it has absorbed with the blood to its development cycle and to be passed on to other individuals. The most important primary malaria vector is anophenoline Gambiae complex (anophenoline arabieansis) that mostly feed on human blood and transfer malaria parasites (de la Fuente *et al.*, 2008). Today, the vector control rarely relies on a single control intervention. Whenever possible, environmental, biological, chemical (insecticides) control measures complement each other known as integrated vector management (van den Berg *et al.*, 2013).

2.2 Diagnosis of Malaria

The various diagnostic tools currently available for identification of *Plasmodium* species in human blood samples include conventional and fluorescence microscopy, immuno-chromatographic lateral flow assays commonly known as rapid diagnostic tests (RDTs),

serology and nucleic acid amplification techniques (NATs) that include PCR and Loop mediated isothermal amplification techniques (LAMP) (Tangpukdee *et al.*, 2009). Out of these tests, the most commonly performed diagnosis of malaria at the primary health care levels is limited to either microscopy or RDTs (Wongsrichanalai *et al.*, 2007b). Whereas the gold-standard microscopic detection of malaria parasites has several limitations in proper diagnosis of malaria.In case of RDTs, the dye-labelled antibody binds to a parasite antigen, and thereafter the resultant complex is captured on the strip by a band of bound antibody that forms a visible line. Microscopy and RDT has limitations, especially in terms of differential sensitivity in different products (Berzosa *et al.*, 2018).

2.1.4 Clinical Diagnosis

Clinical diagnosis is the least expensive, most commonly used method and is the basis for self-treatment. However, the overlapping of malaria symptoms with other tropical diseases impairs its specificity and therefore encourages the indiscriminate use of antimalarial for managing febrile conditions in endemic areas (Ashley *et al.*, 2018). Although highly debatable, this practice was understandable in the past when inexpensive and well-tolerated antimalarial were still effective. The accuracy of a clinical diagnosis varies with the level of endemicity, malaria season, and age group. No single clinical algorithm is a universal predictor. Clinical diagnosis can determine the treatment decision only in children in high-transmission areas. In this situation, a majority of the population is chronically parasitemic and malaria may be concomitant but not the responsible agent of the febrile illness (Amir *et al.*, 2018)

2.2.2 Microscopy

Microscopic examination of Giemsa-stained thin or thick blood smears remains gold standard for the diagnosis of malaria infection. Preferably used in some parts of the world, including Ethiopia where malaria is endemic in primary health care and hospitals as a first-line diagnostic tool (Mfuh *et al.*, 2019). Even though the technique is simple, useful to determine the species and density of parasites, a skillful experienced technician is required to examine and interpret the slides, particularly when identifying parasites at low density or in mixed malarial infection (Vijayalakshmi, 2019).

The microscopic tests involve staining and direct visualization of the parasite under the microscope. Thick smears are 20-40 times more sensitive than thin smears for screening of *Plasmodium* parasites with a detection limit of 10-50 trophozoites/μL set by WHO. Thin

smears allow one to identify malaria species (including the diagnosis of mixed infections), quantify parasitemia, and assess for the presence of schizonts, gametocytes, and malarial pigment in neutrophils and monocytes (Okell *et al.*, 2012). The accurate diagnosis of *falciparum* malaria in an acutely ill patient seeking routine care requires microscopy examination of a Giemsa-stained blood smear. The other is the use of an immuno chromatographic cassette containing monoclonal antibodies to a *P. falciparum* antigen (RDT) (Berzosa *et al.*, 2018). Clinical signs and symptoms alone, though frequently used, can neither differentiate malaria infection from other causes of febrile illness, nor distinguish between *Plasmodium falciparum* and *P. vivax* or other malaria.

In clinical trials, false positive diagnoses lower the apparent efficacy of antimalarial agents, subjecting potentially effective drugs or vaccines to unjustified discarding. False-negative results could lead to overly optimistic outcomes of interventions (unless a follow-up blood smear is possible) or underestimation of the specificity of new diagnostics under evaluation (Wongsrichanalai *et al.*, 2007a). Improving diagnostic accuracy in malaria control systems can be both technically and financially challenging. Continued supervision and support are essential to ensure the sustainability of accurate diagnosis and, thereby, appropriate treatment (Wongsrichanalai *et al.*, 2007a).

2.2.3 Rapid Diagnostic Tests

Rapid Diagnostic Tests (RDTs) offer a useful alternative to microscopy in situations where the reliable microscopic diagnosis is not available. RDTs test is a device that detects malaria antigen in a small amount of blood usually 5-15μL, by immunochromatographic assay with monoclonal antibodies directed against the target parasite antigen and impregnated on a test strip (Organization, 2018). The test results are interpreted by the absence or presence of a colored line or bands on the strip whichcan be obtained within 5-20 min (Wanja *et al.*, 2016).

Commercial tests like RDTs are manufactured with different combinations of target antigens to suit the local malaria epidemiology. The most common malaria antigen targeted and currently commercially available is Histidine-rich-protein-2 (HRP-2) and parasite lactate-dehydrogenase (LDH) (Menegon *et al.*, 2017). Test line configuration and interpretation of RDT results vary with products. Products that incorporate an HRP-2 assay with a pan-malaria pLDH assay are also available. Unlike HRP-2, which often persists in the patient's blood for weeks after successful treatment, pLDH is a more appropriate target for treatment monitoring

(Maltha *et al.*, 2014). However, *Plasmodial* gametocytes also produce pLDH and so a pLDH test may remain positive despite clearance of the asexual parasite forms. One of the advantage of persistent HRP-2 is detecting low-level fluctuating parasitemia in chronic malaria. Both HRP-2 and pLDH-based tests have been used with peripheral and placental blood specimens for the detection of malaria in pregnancy with variable outcomes (Bwire *et al.*, 2019). Overall, WHO puts the minimum detection limits of RDTs up to 50-100 parasite/uL of blood (Mbanefo and Kumar, 2020).

2.2.4 Molecular Techniques

The most commonly used molecular techniques for malaria diagnosis were nested-PCR, conventional PCR, LAMPs, qPCR and multiplex-PCR. Most of these techniques were limited to research purposes and not used for clinical diagnosis except in some developed countries where facilities and other infrastructure were easily available. These techniques are more significantly sensitive than the most commonly clinical used diagnosing tools like microscopy and RDT. Quantitative real-time PCR is the most sensitive TaqMan-based parasite detection and quantification method. This sensitive method can able to detect very low parasitemia in general and nPCR can detect less than 5 parasite/uL of blood (Tedla, 2019). Some of the PCR detection limit factors were the amount or volume of blood and quality of DNA extraction and additionally detects mixed infection and sub-microscopic infection that can't be detected either by microscopy or RDT. In the framework of malaria elimination and control strategies set by WHO, accurate (prompt) diagnosis followed by adequate treatment is very important in detecting early drug resistance for health policy. One advantage of qPCR is the lack of post PCR handling via fluorophores that helps both quantifications and detects up to 0.02 parasite/uL of blood.Such advanced molecular techniques play a great role in successful treatment, especially in appropriate drug efficacy endpoint decision-making. However, some of the major disadvantages of all kinds of PCR are cost perspectives, availability, simplicity and limited to research purposes rather than field and clinical diagnosis purposes, especially in developing countries (Mens *et al.*, 2007).

2.3 Antimalarial Drugs

Antimalarial drug efficacy is assessed through therapeutic efficacy studies (TES and are conducted in a controlled environment in which drug administration is supervised, and the results of microscopic examinations of blood films are validated, and the origin and quality of the drugs are verified (Messerli *et al.*, 2017). TES are prospective evaluations of patients'

clinical and parasitological responses to directly observed treatment for uncomplicated malaria. Studies at regular intervals of the same sites allow for the early detection of drug resistance (Singana *et al.*, 2016).

Artemether-Lumefantrine AL or (Coartem):-The Ethiopian federal ministry of health (FMoH) has started coartem as a first line treatment onwards from 2004 G.C following chloroquine drug resistance for uncomplicated *P. falciparum* malaria infection. AL have common adverse effects of that previously registered by Ethiopian Food and Drug Administration (EFDA) such as vomiting, diarrhea and very less hemolytic effects (anemia). Most African countries use Coartem (AL) as the first-line treatment for uncomplicated *P. falciparum*. Coartem is the combination of artemether and lumefantrine in which both of them are blood schizonticides. The complementarity of pharmacokinetics and dissimilar modes of action enables to provide synergistic anti-malarial activity. Artemether is the shortest half-life artemisinin derivative drug that is rapidly eliminated from plasma within 2-3 hours and act against asexual parasite clearances. While lumefantrine stays longer and eliminated within three to six days that, enabling to provide high cure rate after a short treatment course (Nega *et al.*, 2016). Currently, FMOH was under evaluation of (s) PQ in combination with AL for *falciparum* malaria as a malaria pre-elimination strategies.

Chloroquine phosphate (CQ). Chloroquine is the preferred and first line treatment for *Plasmodium vivax* with long term elimination half- life compared to coartem (AL) (Abreha *et al.*, 2017). In addition to CQ for *P. vivax*, 14 days primaquine used to improve the gametocyte clearance to shorten the life cycle of the infection in both liver and blood stream. Currently, FMOH was under evaluation of CQ in combination with PQ for *Vivax* malaria radical cure set in its goal of malaria pre-elimination strategies.

Primaquine (PQ):- Primaquine phosphate is an 8-amino-quinoline compound that eliminates exo-erythrocytic of *Plasmodium* malaria infection and against mature gametocyte clearance (Shitaye *et al.*, 2018). Thereby, it prevents the development of the blood (erythrocytic) forms of the parasite, which are responsible for relapses in vivax malaria. Primaquine phosphate is also active against gametocytes of *Plasmodium falciparum* and is indicative for the radical cure (prevention of relapse) of vivax malaria with adverse effects of acute hemolytic in patients who are glucose-6-phosphate dehydrogenase deficient (G6PDd). Its elimination half-life is approximately 3.5-8 hours (Xu *et al.*, 2020).

3. MATERIALS AND METHODS

3.1 Description of Study Area

The study was conducted in the health facility of Bambasi district which is on the Gimbi-Assossa road (located 45 km north of Asossa town, capital of Benishangul-Gumuz regional state administration) with an estimated total population size of 70,844, unpublished data from Woreda Admistration. Asossa is located at 666 Km west of the capital city Addis Ababa. Bambasi is one of the 20's districts in Asossa zone, northwestern of Ethiopia, has a longitude and latitude of (9°45′N 34°44′E) respectively (Fig.2). Averagely with an elevation of 1668 meters above sea level and 621 km far away from the capital city, Addis Ababa, Ethiopia. Bambasi has hot and humid weather conditions and a swampy area with a big River called Dabus border in the West Wollega zone, Oromia region used for irrigation and that may create conducive environment for mosquito larval breedings. The geoghraphic location of the study area was produced in Arc GIS 10.7 version (Fig. 2).

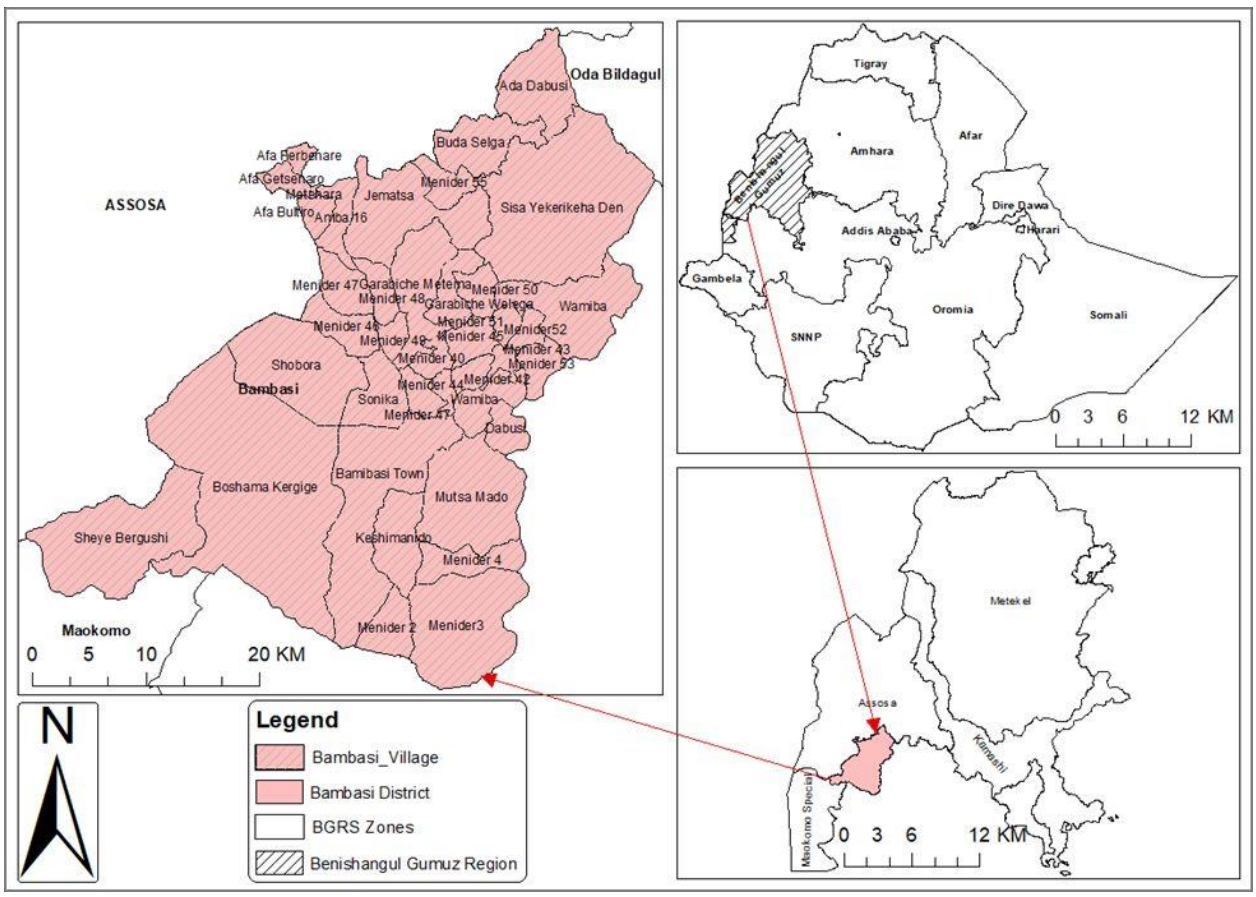


Figure 2: Geographic Location of the study area.

3.2 Study design and Periods

One arm prospective longitudinal surveillance study design was applied during the malaria transmission season from November 2020 to February 2021. It was conducted in Bambasi health center to assess the safety and efficacy of AL + (s) PQ treatment in uncomplicated *P. falciparum*.

3.3 Study population

The population of interest of all individuals living within a catchment radius of the Bambasi health center was considered as a study population. Self-presenting febrile patients of 6 months and above age individuals with a history of febrile in the past 24/48 hours and confirmed with uncomplicated *P. falciparum* mono-infections who meet the study inclusion criteria were considered as study subjects and enrolled into this study.

3.4 Sample Size

The required sample size is defined as the total number of patients who can be assigned a clinical outcome at the end of the study (i.e. adequate clinical and parasitological response (ACPR) or failure due to recrudescence, after confirmation with PCR correction). The calculation is based on an expected proportion of treatment failures in the study population and the desired levels of confidence (95%) and precision (5%). For example, in the case of a medicine with an expected failure rate of 5%, a confidence level of 95% and a precision level of 5%, a minimum of 73 patients should be enrolled. Additional 20% should be added to account for patients who are likely to be either lost during follow-up, withdraw or are excluded after detection of reinfection with PCR correction and this makes 88 patients were required per treatment per site. For single-arm efficacy studies, sample size was determined using classical statistical methods stated below. $N= (z/d)^2 *p (1-p)$, where Z is the critical statistical value at 95% CI (1.96), d=5% desired values and P =5% precision (WHO, 2009). Based on this, 123 patients were enrolled.

3.5 Inclusion Criteria

Patients who meet the all inclusion criteria were enrolled into the study. The following criteria were considered as a study subjects: both sex with age ≥6 months; body weight >5 kg; hemoglobin > 5.0 gm/dL, fever (axillary temperature ≥ 37.5˚c) or having a history of fever in the preceding 24/48 hours patients were recruited. RDT malaria positive and microscopically

confirmed *P. falciparum* mono-infection, with minimum lowest parasite load of 500 parasites/μL and non-pregnant or non-breastfeeding women were enrolled. The patients or guardians who voluntarily signed informed consent to participate in 42 days of follow up were critically considered in the enrollment process.

3.6 Exclusion Criteria

Mixed or mono-infection with species other than *P. falciparum*, hemoglobin < 5.0 gm/dL and history of any antimalarial drug intake within 14 days prior to study recruitment were excluded. Breastfeeding, malnutrition, severe anemic, and pregnant women were excluded. Patients with danger signs and unable to take oral medication or repeated vomiting, another additional co-infection, known hypersensitivity to either antimalarial drugs, and unwilling to follow up were also excluded from the study clinical drug efficacy trials.

3.7 Loss to follow up and patient discontinuation

Loss to follow-up occurs when, despite all reasonable efforts, an enrolled patient does not attend the scheduled visits and cannot be found. Every effort was made to schedule a follow-up visit for patients who fail to return to the study site, especially after administering the study drug. Those patients who cannot be found were classified as lost to follow-up and excluded from the study. Patients who are lost to follow-up but who subsequently return to the study site before day 42 were encouraged to return for check-up visits.

3.7.1 Patient discontinuation

Patients meeting any of the following criteria were withdrawn:-Patients vomiting both initial and replacement doses at any single time in the treatment (i.e. if the patient vomits both attempts to administer the morning dose that would require withdrawal; however, vomiting the initial morning dose but not the morning replacement dose would not require withdrawal). Persistent vomiting, severe side-effects necessitating hospitalization, progression to severe malaria, failure to attend the scheduled visit during the first 3 days will be withdrawn from the study. Voluntary protocol violation like antimalarial (or antibiotics with antimalarial activity) treatment administered by a third party or self-medication with antimalarial (or antibiotics with antimalarial activity) assessed by asking participants or their caretaker at follow-up visits were considered.

3.8 Screening and Enrollment

3.8.1 Screening

Febrile (auxiliary body temperature ≥37.5^{o}C) or a history of fever in the last 24/48 hours and eligible was as per indicated in inclusion criteria . Those volunteer were further screened as per the national malaria guideline, microscopy confirmed *P. falciparum* mono-infection were enrolled. Enrollment was done by a staff member not involved in the clinical assessment of the participant to ensure that consent was freely given. Patients who met the screening criteria and the patient or guardian signed the informed consent form were assigned a unique study number. This number became the patient's identifier in all forms and blood samples from that patient for appropriate tracking every event/record for the case. After obtaining informed consent,each participant was weighed for appropriate drug dosage administration.

3.8.2 Enrollment

Volunteer patients who fulfill the inclusion criteria went through the enrolling process. The health professionals at site of enrolment checked the participants for signs/symptoms of clinical malaria and make a microscopic/MSPP RDTs confirmation from finger prick. Volunteers found to have any of these conditions were treated as per the updated national guidelines, and if deemed necessary has been sent to the next referral link in the health system. If the volunteer fulfills the inclusion criteria, for each study participants, case report forms (CRFs) was completed. Appointment schedule was clearly explained and a follow-up card with a personal identification number also provided. For every volunteer women of child bearing (12-49 years) or who are menstruating; a urine pregnancy test was conducted and if tested positive, excluded. For those enrolled confirmed *P. falciparum,* a venous blood was collected to assess for thin smear to verify parasite species and thick smear to conduct a formal parasite count at day zero.

Clinical data and finger blood sample reassessments were made on days 1, 2, 3, 7, 14, 21, 28, 35, and 42 days. Due orientation was given for those put on 14 days PQ on urine color chart monitoring: Participants was advised to return immediately on any day during the follow-up period if symptoms return and/or if they notice darkening of their urine to stop taking PQ.

3.9 Hemoglobin

Hemoglobin concentrations were measured using a portable spectrophotometer at days of enrollment and other days of visiting appointment on day 3, 14, 28, and 42.

3.10 Pregnancy Test

Pregnancy test was performed for all female participants of with age ranges of 12-49 years during screening for enrolment as per the WHO protocol for primaquine treatment.

3.11 Antimalarial Treatment

All antimalarial drugs were obtained from WHO Global Malaria Program. Participants enrolled in this study were treated as per the 4th edition of the national malaria guideline (FMoH, 2018). Participants with confirmed *P. falciparum* were treated with six-dose regimen of Coartem® given twice daily for three consecutive days plus single low dose of PQ (0.25 mg/Kg body weight). The first doses of the medication was administered in the study health center under direct supervision by the study team either nurse/health officer (HO). While the second dose of day 0 medication was administered 8 hours later at home by the patient or caregiver. Similarly, first doses on each visiting day under direct supervision whereas the second doses of day 1 and day 2, were given 12 hours later, at home by the patient or caregiver. The medications to be given at home was prepared by the study nurse/HO during the clinical visit with proper and clear verbal instruction on when and how to take the medicine.

3.12 Concomitant Treatment

Using standard procedures, health professionals administered supportive treatment to patients as might be necessary: participants with admission axillary temperature >38^{o}C received a standard dose of 10 mg per kilogram paracetamol tablets/syrup every 6 hours needed until the next visit. All patients enrolled in the study were given two additional doses of paracetamol for use at home.

3.13 Efficacy Evaluation Criteria

Primaquine as a radical cure aims to shorten the duration of gametocyte circulation and hence to reduce the probability of onward transmission of malaria, therefore, facilitating control and elimination efforts. It is the only gametocidal agent that reduces the potential transmissibility of malaria infection later on to mosquito and helps in blocking the transmission as a key

component for pre-elimination when given in combination with artemisinin. However, if taken in full doses, may lead to acute haemolysis in some patients with low G6PD (Han *et al.*, 2021). The administration of this drugs has to be under close supervision of health professionals and it's recommended with or after food intake to minimize the side effects. The primary outcome of this study was to monitor the safety and efficacy of AL plus (s) PQ for uncomplicated *P. falciparum* as a routine follow up study in patients age greater than 6 months. Produce also baseline data for the malaria control and prevention program on rate of mature gametocyte clearance in *P. falciparum* infections.

3.14 Safety End Points

The incidence of any adverse event that entails the discontinuation of the medication was assessed during the follow up visit for each study participants.

3.15 Clinical and Parasitological Evaluation

Clinical and parasitological were assessed at enrolment (D0) and the scheduled visit dates; Days 1, 2, 3, 7, 14, 21, 28, 35, 42 and at unscheduled visits as necessary. A standard physical examination with complete medical history, body temperature, demographic information, and contact details were recorded at baseline. Body weight and body temperature was taken at enrolment and subsequent visits. Appropriately calibrated weight scales and thermometer verified before the study begins and checked at regular intervals were used to measure (Stepniewska *et al.*, 2004). Digital thermometer with a precision of 0.1°C was used to measure temperature. The same route of measurement were used throughout the study to take body temperature. In case, the read was below 36 °C, it was repeated. Participants should remove excessive clothing and young children should only wear undergarments while being weighed. Weight of the patients were recorded to the nearest kilogram. For under-five children, the left mid-arm circumference was measured to the nearest centimetre measurement. For indication of severe malnutrition, pitting oedema on the dorsal surface of both feet was assessed by thumb pressure for 3 seconds (Teklemariam *et al.*, 2017). The clinical and parasitological outcomes of antimalarial treatment according to the latest guidelines of WHO protocol (Wang *et al.*, 2020). Accordingly, all *P. falciparum* patients were classified as having an early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR) (Olumese, 2006).

3.16 Sample Collection

A finger prick blood samples (approximately 350-400 uL) were collected for smear microscopy, hemoglobin measurement and dry blood spot (DBS) from all recruited patients during the 42 days follow-up visits of period. Blood samples were simultaneously spotted on Whatman 3 MMR filter paper, dried overnight, and stored individually in sealed plastic bags with a desiccant at 2-8 ^{0}C until before use for molecular analysis.

3.17 Laboratory Examination

3.17.1 Blood Smear Microscopy

Blood smears were taken from all participants, and thick and thin blood smears were prepared on the same slide to detect parasites at all time-points during the 42-day follow-up period. Two uL of blood and 6 uL of blood were collected to prepare thin and thick blood film, respectively. On day 0, in order to rapidly confirm adherence to the lowest parasite density considered for enrollment. Initial patients screening was made by 10% Giemsa-stained thick blood smear for 30 minutes and after counting at least 1 parasite for every 6-8 WBCs, which corresponds to approximately 1000 parasites/μl. The second slide was stained slowly to be examined later by an experienced technician to provide definitive parasite count and speciation. Parasite densities were calculated from thick blood smears by counting the number of asexual parasites against 200 WBC. If the count is <10 parasites/200 WBC, at least 500 WBC were counted. A thick blood smear was declared negative if no parasites are seen in 1000 WBCs and if mixed infection has been seen in 100 fields on thick film as exclusion criteria. The formula presented below was used to determine parasite density.

$$\text{Number of parasite density} = \frac{\text{number of parasite counted x (6000-8000)}}{\text{Number of leukocytes counted (approximately) 200}}$$

3.18 Follow-up Progress of the Study

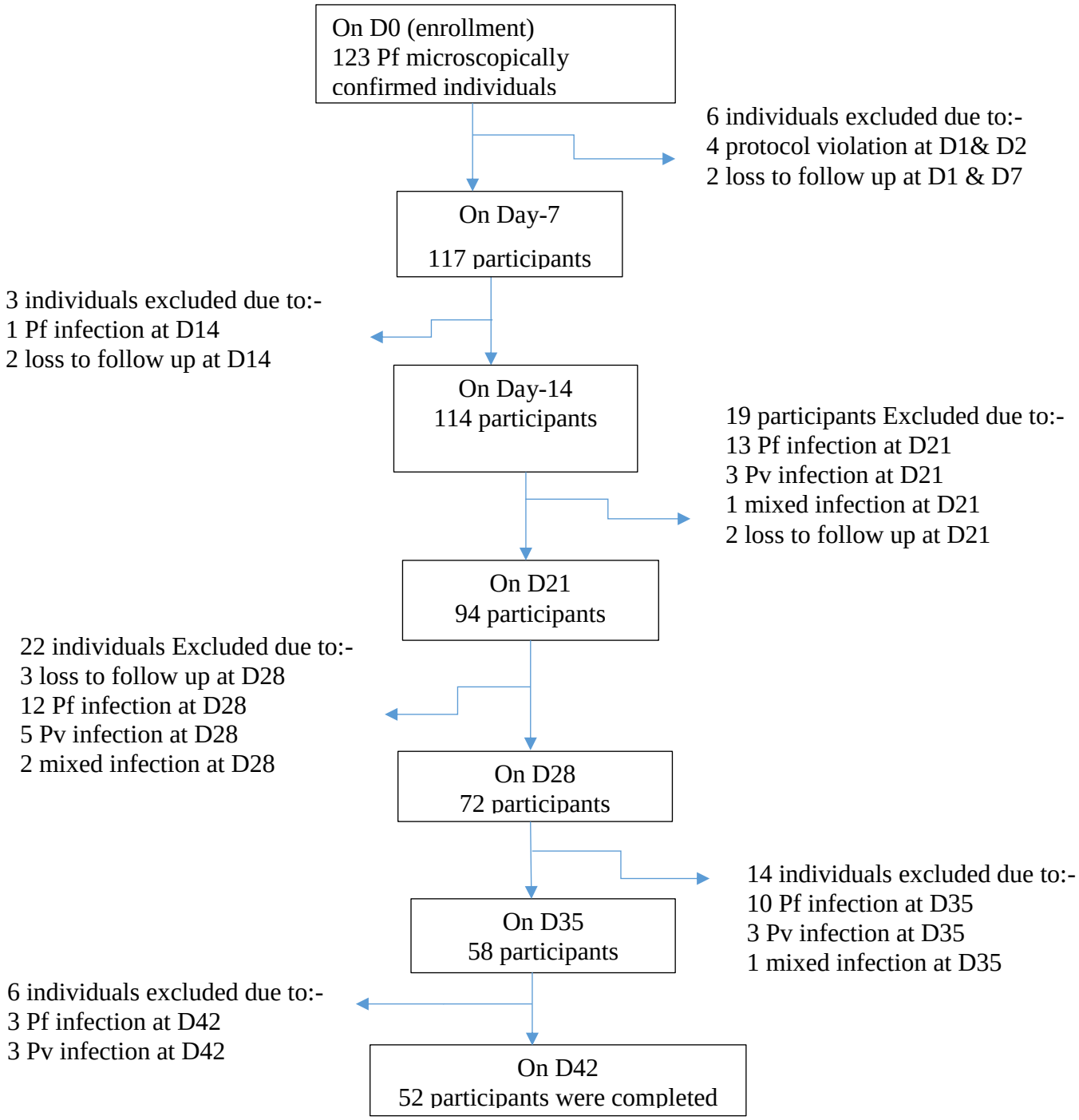


Figure 3: Flow chart of TES study follow up progress in *P. falciparum*, Bambasi, November 2020 to February 2021.

3.19 DNA ExtractionProcedure

Plasmodium DNA was extracted from dried blood spots using Chelex/Saponin extraction method. Approximately 6 mm diameter of the DBS were punched out from blood spot and placed into 1.5 mL microcentrifuge tube in which 820 uL (0.5%) saponin dissolved in PBS solution (BCBS6399V, Germany) was added and incubatedovernight on shaker with 450 tilts per minutes. Saponin was aspirated and 1 mL of Phosphate buffered saline (1x PBS) solution was added and incubated for 30 minutes at $+4^0$C and 30 minutes on shaker with 650 tilts /minute speed. The PBS was aspirated and 150 uL of (6%) Chelex (64065396, US.A) dissolved in molecular grade water was added. Then, incubated in a water bath already set on 97^0C four times for 7 minutes and in between each interval, incubated on ice for 1 minute on a shaker. This step helps to relief from heat pressure. Finally, centrifuged for 5 minutes at 14000 rpm and 80 uL of the supernatant solution of DNA was eluted for PCR analysis.

3.20 Molecular Detection of Parasite Species

Parasite detection was performed targeting 18 small ribosomal unit rRNA genes. After DNA extraction for 123 sampes of baseline, molecular detection of P*lasmodium* species was performed using Snounou nested PCR technique. In a primary PCR reaction, 5 uL of the extracted parasite DNA template was added in 20 uL of PCR cocktail targeting *Plasmodium* gene. Whereas the secondary PCR reaction was run using 2 uL of the primary (N1) PCR product as a template and 23 uL of PCR cocktail containing specific set of primers targeting *P.falciparum* and *P.vivax* respectively (Table 1). The PCR cocktail contains the following components with their final concentration in 1 reaction: Reaction Buffer B 1x of (5x), Separate $MgCl_2$ solution 2 mM, dNTPs 0.25 mM each, forward and reverse primer (0.25 mM each), Taq polymerase 1 U/reaction.

The PCR (Bio RAD T100^{TM} Thermal Cycler) was run at the following thermal cycler conditions: The cycling condition for nested-1 was initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and initial extension at 72°C for 1.5 min with a final extension step at 72°C for 10 minutes. The conditions for nested-2 were identical to the Nested-1 except the number of cycles, 30 cycles instead of 35 (Oyedeji *et al.*, 2007). 2% agarose gel (lot # 104917) was prepared in 0.5 TBE buffer, lot # 2064787, UK) and stained in 3 uL of 1 % ethidium bromide solution (E8751-25G, US.A). Gel electrophoresis resolution was run at 120 V, 400 mA for 1 hour to separate the expected size of *P. falciparum* at 205 bp and *P. vivax* at 120 bp respectively, and cross-referenced with 100 bp ladder (100 DNA Ladder, lot # 0531608 England). The gel was visualized using Bio Rad gel doci x-ray (UV-Bio Rad Universal Hood II, lot # 721BR01632, US.A).

Table 1: List of primers used in nested PCR with their sequence 5' - 3'

18S Snounou assay	**Primer Name**	**Sequences 5' - 3'**	**Length (bp)**	**Amplicon size(bp)**
N1 F & R Primers	rPLU5	CCTGTTGTTGCCTTAAACTTC	19	1200
	rPLU6	TTAAAATTGTTGCAGTTAAAACG	23	
N2 F & R Primers	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	30	205
	rFAL2	ACACAATAGACTCAATCATGACTACCCGTC	30	
N2 F & R Primers	rVIV 1	CGCTTCTAGCTTAATCCACATAACTGATAC	30	120
	rVIV 2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	30	

Sources:(Snounou and Singh, 2002).

3.21 MSP2 *P. falciparum* Genotyping

In a malaria co-endemic area, the parasite recurrence was common and in order to differentiate a recrudescence (same parasite strain) from a newly acquired infection (different parasite strain) a genotype analysis must be conducted. This may be depends on the extensive genetic diversity among the malaria parasite genes of merozoite surface protein-1 (*msp1),* and merozoite surface protein-2 (*msp2).* Following this, the genotypic profiles of pre-and post-parasite strains must be compared to differentiate recrudescence from new infection. In this study, the most common recommended *msp2* was performed.

The primer sequences of MSP2- primary reaction according to Foley et al. 1992 and secondary reaction were according to Falk et al. 2006 with cycling conditions of N1 x34 and N2 x36 respectively. The MSP2 primary and secondary reaction primer concentration were: - dNTPs 10 uM, Mgcl2 solution 25 mM, 10 uM each generic primers and allelic specific primers for secondary reaction (Fc27, 3D7 and S1-tail fw) were 5 uM respectively whereas same in both reaction and enzyme and buffer were 5 units and 5x respectively. 2 uL of DNA template were mixed with 13 uL master mix in primary reaction to make the total volume 15 uL and whereas 1 uL of the amplified DNA was mixed in 14 uL secondary reaction. Then, 10 uL of the PCR product was run for 1 hour at 120 V on 2 % agarose gel stained in 3 uL of 1 % ethidium bromide solution and read under Bio-Rad-UV illuminator. Finally, obtained data was interpreted based on their molecular sizes (bp).

Table 2:List of Primers and their sequences used for msp2-Pf PCR- genotyping

Assay	**Name**	**Sequences 5' - 3'**	**Size (bp)**
Primary reaction	msp2-S2 fw	GAAGGTAATTAAAACATTGTC	
	msp2-S3 rev	GAGGGATGTTGCTGCTCCACAG	
Secondary reaction	Fc27	GCATTGCCAGAACTTGAA	262-668
	3D7	CTGAAGAGGTACTGGTAGA	259-727
	msp2-S1Tail-fw	GCTTATAATATGAGTATAAGGAGAA	

3.22 Ethical Consideration

Ethical approval for the study were both granted from Ethical Boards of the Adama Science and Technology University, Adama, Ethiopia and Armauer Hansen Research Institute, Addis Ababa, Ethiopia and the National Research Ethics and Review Committee on protocol number of PO/23/20 for a duration of October 30, 2020-21. Patients who met all the enrolment criteria were given a personal identification number and received treatment only after the study had been fully explained to them and they had willingly provided informed consent. Written informed consent has been sought from participants 18 years and above, parental/guardian written informed consents were obtained for children whose ages were 18 years and less. For children between the age of 11 and 17 years, verbal assent has been taken in addition to their parent/guardians' informed written consent.

3.23 Data Analysis

All clinical and parasitological data including the adverse log events were well documented and entered in to AHRI Redcap data management system. Then, all data selected for analysis were cleaned and exported into excel and statistical package for social sciences (SPSS) designed by International business machines corporation (IBM) software version 22 for data analysis. Both the clinical and parasitical obtained data were analyzed using frequency and chi-square. Chi-square was used to compare proportions between the two method by setting level of significance at 5 % (=0.05) during the analysis. The other statistical analysis where performed using WHO Kaplan-Meier-method.

4. RESULT

4.1 Socio-demographic Characteristics of the Study Participants

Among the 123 uncomplicated *P. falciparum* study participants, 60% were male, whereas 40% were female participants with an average age of 18 years (ranging from 3 to 62 years) with a standard deviation of 13.6. The mean body weight of study participants was 40.6 (ranges from 12-74) with a standard deviation (sd) of 15.3. The mean axillary temperature was 38 (ranges 36-40) with a standard deviation of 1.2 and with the geometric mean of 14099 (ranges 3330-40000) parasitemia summarized in Table 3.

Table 3: Sociodemographic Characteristics, Bambasi.

Characteristics	**Number, %**
Sex	
Male	74 (60%)
Female	49 (40%)
Age category in yrs (0.6-5)	6 (4.9%)
Age category in yrs (6-14)	71 (57.7%)
Age category in yrs (>15)	46 (37.1 %)
Mean weight (Kg)	40.6
Axillary temperature (^{0} C)	38
Mean Hgb (g/dl)	11
Mean parasitic (p/uL)	31,592

Keys: NB: Age category in years, Number (n), Percent (%), Mean body weight (Kg), Ave. body temp. (^{0}C), Heamoglobin concentration (g/dl) and mean parasitic per uL of blood (p/uL). The mean heamoglobin concentration was in the normal range set by WHO in all age group. Most of the study participants were febrile at the day of enrollment in all age group. The average asexual parasitic density were also high in all age group and with normal range of mean Hgb concentration.

4.2 Treatment Outcome

The therapeutic efficacy of the drugs was classified as per WHO treatment guide line. The number of individuals in each age classification is not proportional.

Table 4: WHO efficacy treatment outcome, Bambasi, November 2020 to February 2021.

Microscopic failure					**nPCR confirmatory test**
Outcome	Age group (years) (n, %)			Total (N=109)	(N=105)
	0.6-5 (N=4)	6-14 (N= 64)	>15 (N=41)		
ETF	0	0	0	0	0
LCF	1 (0.9%)	2 (1.8%)	2 (1.8%)	5 (4.6%)	4 (3.8%)
LPF	2 (1.8 %)	36 (33%)	14 (12.8 %)	52 (47.7%)	50 (47.6%)
ACPR	1 (0.9%)	26 (23.8%)	25 (22.9%)	52 (46.8%)	51 (48.6%)
Total analysis	4	64	41	109	105
Loss to follow-up	2 (1.6%)	8 (6.5%)	4 (3.2%)	14 (11.4%)	14 (11.4%)
Total	6	72	45	123	119

Keys: Early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), adequate clinical and parasitological response (ACPR).

4.3 Heamoglobin Assessment

In all age category of the study participants, the mean of Hgb concentration is in the normal ranges in both sex as per the WHO standard classification. The treatment of *P. falciparum* with a single dose (low conc.) of PQ in combination with AL has no serious anemic effects on the study participants. Summarized in table 5.

Table 5: Average hemoglobin concentration stratified by age group and sex, Bambasi district, November 2020 to February 2021.

Days	**Age group (years)**			
	0.6-5 yrs	6-14 yrs	>15 yrs	Total
	Hgb	Hgb	Hgb	Hgb (g/dL)
D0	11.5	12.9	13.1	12.5
D3	10.9	12.1	12.4	12
D14	11.5	12.8	13.5	13
D28	12.8	13.5	13.3	13.5
D42	12.8	13.6	13.6	13.3

Keys: Day zero (D0), Day one (D1), and similarily for others.

4.4 Parasite Clearance Rate

Proportion of patients with parasitemia and geometric mean of parasitemia per uL of blood over the follow up period of 42 days in the standard treatment of AL+ (s) PQ for *P. falciparum* arm at Bambasi district, 2020 (Fig. 4).

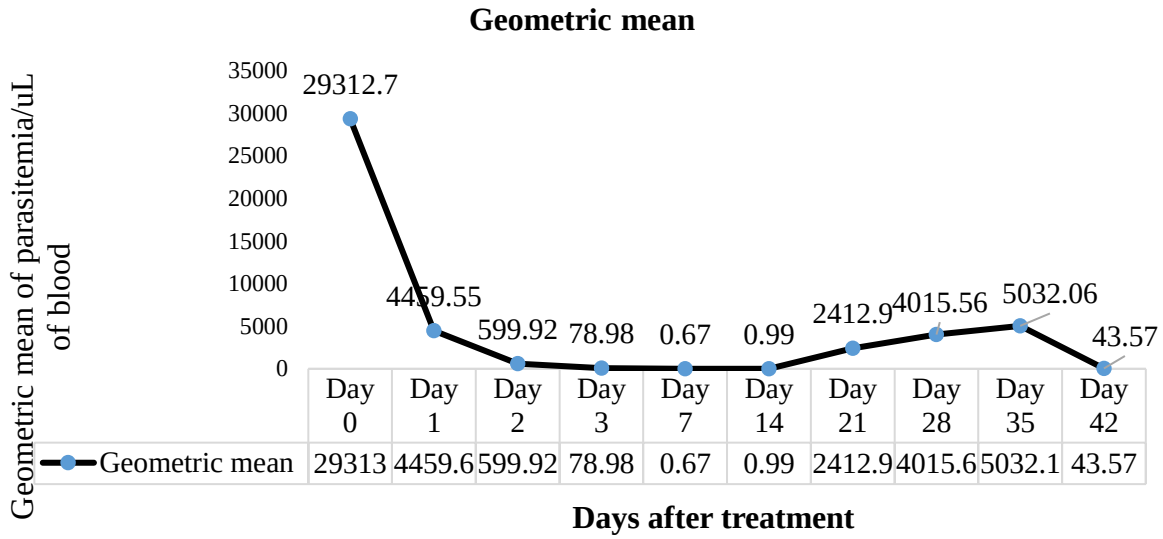


Figure 4: Average rate of asexual parasite clearance, Bambasi district, November 2020 to February 2021.

As shown in Fig. 4 above, the mean parasitic density was exponentially decreased within the first two days after treatment. About 90% of asexual parasite was cleared at day 3 except one individuals lost follow up without parasite clearance after day 7 visits. Parasite re-ocurrence seen on one study participant two weeks after treatment initiation and onwards. However, most of the parasite recurrence was in between day 21-28 during follow-up periods.

4.5 Gametocyte Clearance Rate

Proportion of patients with geometric mean of gametocyte per uL of blood over the follow up period of 42 days in the standard treatment of AL+ (s) PQ for *P. falciparum* arm at Bambasi district, 2020. The gametocyte was exponentially decreased and cleared in between D3 and D7 visits after treatment initiation. Once it's cleared and not detected during the D42 of visits that may be sub-microscopic and possibly detected by molecular techniques (nPCR) Fig. 5.

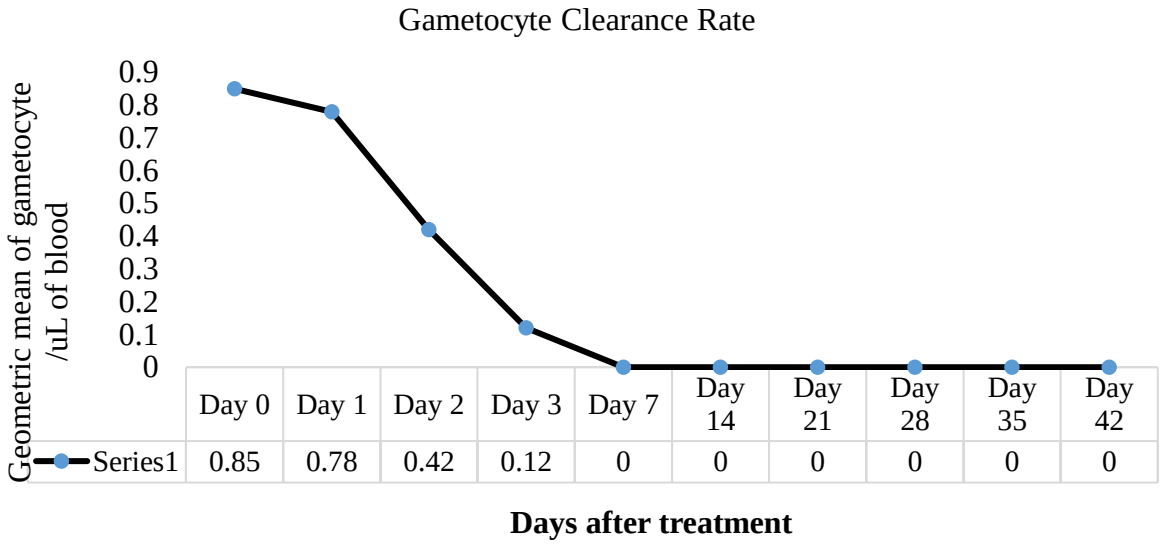


Figure 5: Average rate of gametocyte clearance, Bambasi district, November 2020 to February 2021.

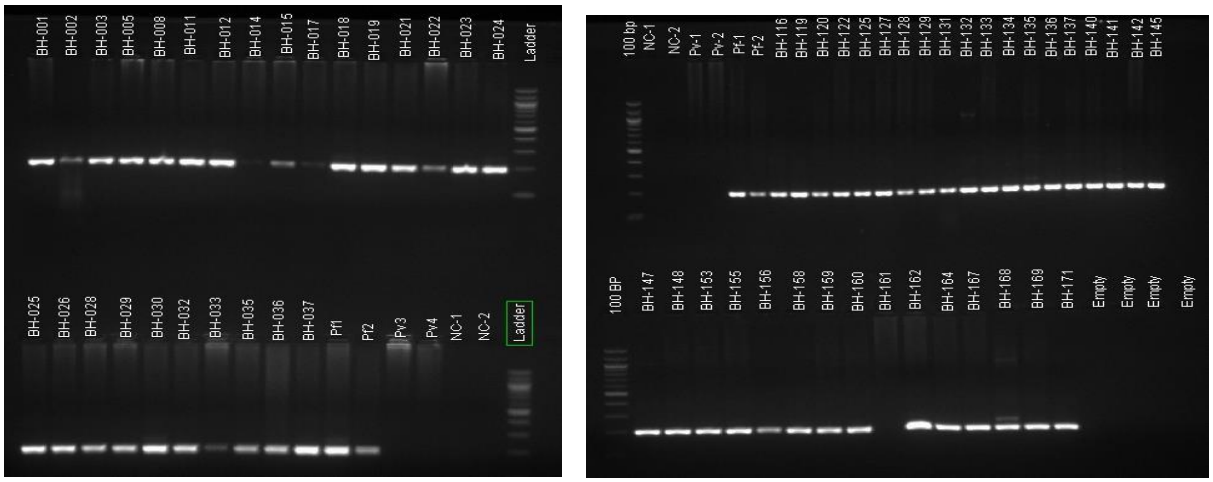
4.6 PCR-Confirmatory Test at baseline

The PCR confirmatory test was performed using previously discribed pair of primers (Snounou *et al.*, 1993).

Table 6: Microscopy and nPCR-results at baseline, Bambasi district, November 2020 to February 2021.

Test proportion at D0	Category	N	Observed Prop.	Test Prop.	Sig. (1-tailed)
Microscopy result	Pf	123	1.00	0.05	0.00
Total		123	1.00		
nPCR	Pf	106	.86	0.05	0.00
	Mixed	17	.14		
	Total	123	1.00		

The proportionality test of the two method agreement was set at 95% CI with 5% tolerance. Based on the analysis, 17 individuals (14 %) were examined with mixed infections that were mis-speciation at the day of enrollment using the two conventional diagnosing techniques of microscopy and RDT respectively (Table 6). The nPCR results tested for both *P.falciparum* and co-infection *Vivax* were attached below. As indicated on gel picture (Fig. 5), both gel tested for mono-infectivity of *Pf (A),* whereas two known clinical samples were used as positive control represented as *Pf1* and *Pf2*. Similarly, for *P. vivax* infection test (B), positive control represented as *Pv1* and *Pv2* and none template control (NTC) were also run in duplicate. All the patients' samples were run and the expected band size of *Pf* at (205 bp) and *Pv* (120 bp) were cross-referenced with the 100 bp DNA ladder.



A B

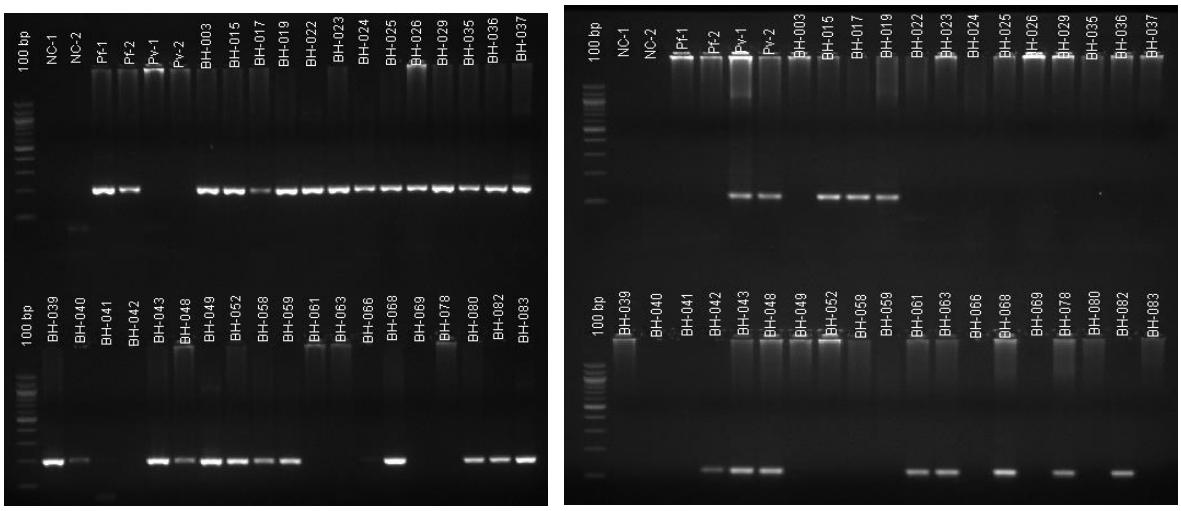
Figure 6: Gel-electrophoresis result of nPCR confirmatory test at baseline, Bambasi district, November 2020 to February 2021.

4.7 PCR-Confirmatory Test of Parasite Recurrence

Table 7: Proportion of microscopic method with nPCR at the days of parasite recurrence, Bambasi district, November 2020 to February 2021.

	nPCR result at days of parasite recurrence				
	Neg	*Pf*	*Pv*	Mixed	Microscopy
Pf	2	32	0	6	40
Pv	2	0	11	0	13
Mixed	0	0	1	3	4
Total	**4**	**32**	**12**	**9**	**57**

The nPCR results were significantly different from microscopic results in both speciation and detection of mixed infection at the day of parasite recurrence (Table 7) and the electrophoresis result was stated below in Fig. 7. The first gel picture (A) was tested for *Pf* nPCR conformity whereas the second right-hand side (B) was tested for *Pv* co-infection. As indicated on gel picture (Fig.6), two known clinical samples used as positive control represented as *Pf1* and *Pf2*. Similarly for *P. vivax* infection known positive control represented as *Pv1* and *Pv2* and none template control (NTC) were also run in duplicate. All the patients' samples were run and the expected band size of *Pf* (205 bp) and *Pv* (120 bp) were cross referenced with the 100 bp DNA ladder.



A B

Figure 7: Gel-electrophoresis of parasite recurrent tested for both Pf (A) & Pv (B), Bambasi district, November 2020 to February 2021.

NB: BH- represents Bambasi Health Center used for sample identification (sample code).

4.8 Adverse Effects of Study Drugs

All information related to the drug used in treatment arm side effects were collected via self-reporting and by interviewing during each follow-up days of visits.

Table 8: Age and sex distribution of adverse effects of standard AL + (s) PQ, Bambasi district, November 2020 to February 2021.

Adverse effects	Sex	(0.6-5) yrs. n=6	6-14 yrs. (n=71)	>15 yrs. (n=46)	Sub-total	Total episodes
Fever	F	2 (1.6%)	5 (4%)	3 (2.4%)	10 (8%)	21.8%
	M	1 (0.8%)	13 (10.6%)	3 (2.4%)	17 (13.8%)	
Chills	F	1 (0.8%)	3 (2.4%)	3 (2.4%)	7 (6.7%)	14.7%
	M	(0%)	7 (5.7%)	3 (2.4%)	10 (8%)	
Headache	F	(0%)	9 (7.3%)	5 (4%)	15 (11.4%)	35%
	M	(0%)	14 (11.4%)	15 (12.2%)	30 (23.6%)	
Nausea	F	1 (0.8%)	1 (0.8%)	(0%)	2 (1.6%)	8.9%
	M	(0%)	7 (5.7%)	2 (1.6%)	9 (7.3%)	
Vomiting	F	1 (0.8%)	1 (0.8%)	(0%)	2 (1.6%)	6.5%
	M	(0%)	6 (4.9%)	(0%)	6 (4.9%)	
Abdominal pain	F	(0%)	(0%)	(0%)	(0%)	3.2%
	M	(0%)	3 (2.4%)	1 (0.8%)	4 (3.2%)	
Weakness	F	1 (0.8%)	2 (1.6%)	3 (2.4%)	6 (4.9%)	9.8%
	M	(0%)	3 (2.4%)	3 (2.4%)	6 (4.9%)	

No serious adverse effects of AL plus single dose PQ throughout the 42 follow-up days of visits were recorded. Overall, study participants reported few common malaria infection signs and symptoms side effects during the visits and frequently reported previously with AL as indicated in above Table 8.

4.9 PCR-genotyping

All paired samples of 43 *P. falciparum* showen treatment failure were genotyped for msp2 loci (3D7 and Fc27 alleles) to differentiate recrudescence from new infection. The msp2 result interpretation was based on the visual inspection (Fig. 7), but, there are other techniques. These are; capillary gel electrophoresis which mostly used in differentiating very close molecular sizes (bp) and gives a better resolutions. The other is software based base pair estimation in which the gel picture fed into the software and gives us the estimated size of the target product length. Most of the products were in the base pair range of 300 bp-650 bp of the two loci tested.

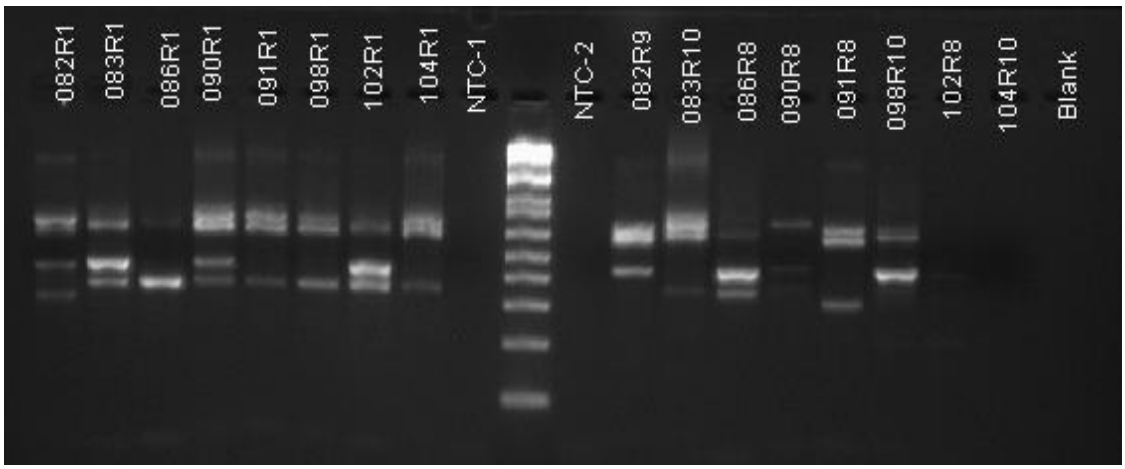


Figure 8:MSP2- PCR-genotyping gel-electrophoresis result for *P.falciparum*.

NB: R1 represents sample at base line (enrollment) whereas R8, R9 and the like are round visits of x-days of post treatment those shown parasite recurrence.

5. DISCUSSION

Recently, WHO recommends a single low-dose PQ in addition to ACT combination therapy to treat *P. falciparum* infections in a malaria low co-endemic settings for the transmission blocking (WHO, 2021). This reduces the mature *P. falciparum* gametocytes carriage that may halts the onward transmission of malaria parasite. Radical cure of malaria parasite normally consists of a blood-stage schizontocidal course of first-line treatment and a course of primaquine to eliminate hypnozoites of co-infections as antirelapses therapy (Miranda *et al.*, 2014). In combination with primaquine, ACT synergistically shortens the circulation of gametocyte to block the transmission dynamics of the parasite between human to female anopheline mosquito. The combination therapy (AL) helps to clear parasitemia, symptoms related to malaria and prevent recrudescence, especially in malaria co-endemic transmission settings.

In this study, microscopically 123 *P. falciparum* were enrolled in in-vivo therapeutic efficacy using RDT as a mono-species confirmatory test and followed for 42 days of visits. Later on, the proportion of concordance and discordance of the two methods were 86% (106/123) and 14 % (17/123) respectively at baseline. This is statistically significantly different at 5% of tolerance of proportionality test. Similarly at the parasite recurrence, their measure of agreement at Kappa value ($P < 0.05$) shows the significant difference between the two methods in specific parasite species identification. Overall, those mixed infections were mistreated due to the drawbacks of detecting minority clones/ mis-speciation of the techniques. In this study, misdiagnoses may be due to the low sensitivity of the method, and the parasite density of co-infecting *P. vivax* is below the detection limit or morphological identity during enrollment. The poor sensitivity of diagnostic tools lead to improper treatment that leads to incomplete parasite clearance that leads to either relapse or early recrudescence or new infection especially expected high in malaria co-endemic settings (Woessner, 2015).

In the study, the mixed infections were contributed more to the treatment failure. The majority of them, 64.7 % (11/17) were faced the reappearances of the parasite (recurrence) and out of this, (8/11) were parasite recurrence with *P. vivax* co-infection three weeks after treatment. These indicating that, a single low dose treatment of primaquine with AL may not properly clear the asexual gametocyte carriage of the individuals. This helps the mature schizonts to have an opportunity to rupture and back to the blood stream (White, 2011). Mature gametocytes can persist after asexual forms of parasite clearance and can be detected by PCR that leads to

incorrect treatment failure misclassification. The other possibility may be primaquine's low dosage slow action on pre-erythrocytic and erythrocytic forms of *P. falciparum* and *P. vivax* co-infections, resulting in recurrent parasite infections. Overall, almost half of them were show treatment failure.

Therefore, the conserved and highly polymorphic variable region of merozoite surface protein-2 called msp2 genotyping marker was performed to distinguish between recrudescence and new infection. The same allelic families as a recrudescence with less than (20 bp differences) and different alleles as new infection with more than (20 bp differences) were considered during the classification (WHO, 2008). The molecular genotyping (msp2) at baseline and post-treatment recurrent *P. falciparum and* mixed infections were used to distinguish recrudescence from new infections to estimates the PCR corrected as optimal efficacy endpoints. In the presnt study, in addition to base pair differences, the following rules accounted during the molecular data interpretation. The parasite recurrence can be potentially classified into four categories depending upon the degree of allelic matching in addition to base-pair relatedness classification: (i) all alleles at baseline and recurrent are identical; (ii) some alleles are missing in recurrent parasites; (iii) the recurrent parasite contains alleles identical to baseline with additional or new clones that may be not seen at baseline; (iv) all alleles in the baseline and recurrent parasites are different which is considered as new infection whereas (i-iii) were an indicators of recrudescence (Snounou and Beck, 1998). However, some investigators consider the category of step (iii) mentioned above as new infection due to the appearances of new alleles different from baseline (Cattamanchi *et al.*, 2003).

Most of *P. falciparum* genes shows extensive genetic polymorphism in merozoite surface protein-1, 2 and glutamate rich protein (*glurp*) marker gene in different geographical location of malaria-endemic areas in Ethiopia (Mohammed *et al.*, 2017). The PCR-correction efficacy endpoint over estimates recrudescenses, especially when the trials are conducted in high malaria transmission settings with frequent new infections. Even a perfectly efficacious drug could have a high apparent treatment failure rate as new infections would be mistaken as drug failures in such areas. In the present study, all microscopically shown treatment (*Pf* and Mixed) 41 paired *P. falciparum* samples were msp2 genotyped and of this, 40 paired samples were successfully amplified. Whereas one nPCR positive sample was unresolved (no signal) at recurrent days with low parasitemia (80 p/uL). One of the main reasons may be the poor DNA extraction quality, which leads to below the detection limit of PCR performance or may be

sequestered even if DNA was extracted with high yield. Other may be due to the low parasitemia that results in low DNA yield or some persistent PCR inhibitors that may compromise its performance (Mugittu *et al.*, 2006).

In this findings, based on the msp2 genotyping result, the PCR-uncorrected treatment failure was 44.8% (CI 95%: 35.6-55.2%) with a cumulative success incidence rate of 55.2% (CI 95%: 44.8-64.4) respectively with total censored=14 and with 96 total patients per-protocol (TPP). While the PCR-corrected treatment failure was 14.3% (8.3-24.2%) with the success incidence rate of 85.7 % (75.8-91.7 %) at 95 % CI respectively. However, this efficacy success of the study drug was below the limit set by WHO which is 90%. Statistically, the difference between conventional microscopy efficacy endpoint and PCR correction was 30.5% at 95% CI indicating the role of molecular techniques in better estimation of efficacy assessment endpoint. This implies that, in efficacy assessments, especially in high malaria transmission settings, the longer follow-up periods, the more likely patients get infected with malaria parasite to acquire new infection. This findings is not in line comparably with the published data of similar studies. However, a recent report in our country, Chewaka, South western Ethiopia, conducted in moderate transmission setting shows the high efficacy of AL alone both PCR uncorrected and corrected was more than 94% (Abamecha *et al.*, 2020).

Similarly, in Tanzania, AL with or without a single low dose (0.25mg/Kg) of PQ were compared and in both regimens, similar PCR-ACPR was seen. Overall, no gametocyte carriage after treatment in all acute uncomplicated *P.falciparum* at 28 days of follow-up visits (Mwaiswelo *et al.*, 2016b). In South Africa also, the single dose of PQ + AL followed for 42 days of visits was assessed and in line with the WHO efficacy threshold (Barnes *et al.*, 2020). Authors recommended the deployments due to its safety and tolerability perspectives without G6PDd test in low transmission settings.

In this findings, the multiplicity (complexity) of infections were seen that indicate, in a single individuals, more than 2 distinict parasite alleles were detected. The multiplicity of infections (MOI) in a host depends on the transmission intensity, acquired immunity and other host factors. For example, in high transmission settings, and individuals can have approximately an average of more than three parasite clones whereas in regions of intermediate or low settings, one or more parasite clones can be seen (Schoepflin *et al.*, 2009). Therefore, based on the genotype result of the present study, except few patients with monoclonal infection, in most of them, polyclonal infections were seen, indicating that, the area was high transmission setting.

However, some of the currently used genotyping tools drawbacks were the difficulty of detecting all minority parasite clones and the discrimination power of very close (similar) molecular size parasite clones using agarose gel electrophoresis (Juliano *et al.*, 2010), which can be resolved by capillary electrophoresis (Liljander *et al.*, 2009).

In our assessment, the asexual parasite clearance and gametocyte were exponentially decreased and cleared from all individuals at Day 3 except one participants loss to follow up at day 7 without parasite clearance. This finding suggests that, a potential risk of cross-border *falciparum* resistance marker to artimesinin derivatives from Sudan (Mohamed *et al.*, 2020), that escale up the treatment failure since the area shares border with Sudan and refuge camp in the area. Recently, in Rwanda, in patients infected with *falciparum* treated with artimesisin combination thereapy (AL) alone, 12.8% sample positivity were observed after day 3 of treatment initiation (Straimer *et al.*, 2021). However, among individuals living in high endemic areas, immunity to malaria develops generally through repeated infections (McCallum *et al.*, 2008). In this findings, its unclear that, which factors contribute for the treatment failure faced. However, several factors affect the antimalarial treatment responses such as drug concentration, host malaria specific immunity, pre-treatment parasite density and intrinsic drug-related effects that may alter or increase the effects of a drug on the parasite (Toure *et al.*, 2018). Malaria-specific immunity plays an important role in malaria parasite clearance. In addition to this, the half-life of the drug used may also play a great role in treatment failure. The shorter the half-life, the more probability of occurring treatment failure. Because, as the residue of the drug clear from the plasma, the more probability of getting recrudescence or new infections is high. However, AL has a short clearance rate from blood and PQ also has no longer half-life. The other may be possibility of reinfection occurrences after day 7 onwards as indicated in WHO updated treatment protocol.

In our assessment, an individuals with minimum heamoglobin concentration of 6.8 Hgb (anemic range) enrolled at day zero and treated with AL + single dose of PQ showed a slight increase in Hgb conc. a week after treatment. However, it shows dramatic increment after day 14 upto day 42 with a minimum Hgb of 9 g/dL, while others have a maximum Hgb range of normal (16-18) (Table 5). Most of the adverse reactions recorded in the present study were those previously registered by EFDA and common in other similar studies of AL alone with a low percentage. In most of the patients, the febrileness, headache and other adverse events were absent in the first two days after treatment initiation following the exponential decrease of

parasetimia. Therefore, we can conclude that, no serious anemic effects on the study participants were registere during the treatment of *P. falciparum* with a single dose (low conc.) of primaquine in combination with AL. Overall, primaquine is safe in all study individuals in the area with the exception of inclusion criteria and regardless of G6PDd test.

6. CONCLUSIONS AND RECOMMENDATIONS

Adding single low dose-PQ to standard AL regimen is tolerable, safe regardless of G6PDd test and this study supports deployments as WHO recommended in low transmission settings in all districts of Ethiopia. However, delayed parasite clearance is observed in light of the single PQ use. We recommend a close follow up and further study. A systematic molecular surveillance to assess the possible mechanisms of artemisinin-tolerate parasites in the area is advised.

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Appendix 1: Consent form (English version)

Name of study participant ---------- Age---------- Sex----------

Physician Name---------- Site /Health center ----------

I have been informed about a study that plans to investigate therapeutic efficacy study in Benishangul-Gumuz Regional state, Bambasi health center entitled "Therapeutic efficacy of Artemether-Lumefantrine (AL)plusSingleDose Primaquine(PQ)for the treatment of uncomplicated *Plasmodium falciparum*in a Co-endemic setting (Bambasi)" which will help in investigating the extent to which malaria is a public health problem in the area. This study could contribute to in recommending the use of appropriate control measures that can minimize the transmission of the disease in the area.

For the study I have been requested to give a drop of blood from my finger and children under my guardian. They told me that experienced health professionals according to the established aseptic procedure would do the blood collection on to glass slide by finger picker (lancet). I have been informed that if positive result is observed treatment will be given to children and me by using the standard drug regimen. Based on this, I have agreed to continue the examination with my children and me too. The investigator also informed me that if I want all the laboratory results would be kept confidential.

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 and cooperates at my will in the course of the conduct of the study.

Name (participant) ----------Signature ----------Date ----------

Name (investigator) ----------Signature ----------Date ----------

Name (Witness) ----------Signature ----------Date ----------

Appendix 2: Consent form (Amharic version)

ለወላጅ/አሳዳጊየስምምነትመግለጫቅጽዕድሜያቸውከ6ወር -11 አመትለሆኑተሳታፊዎችየጥናቱርእስ፡-*In-vivo* Therapeutic efficacy of Artemether-Lumefantrine (AL)plusSingleDosePrimaquine(PQ)forthetreatmentofuncomplicated*Plasmodiumfalcipa rum.*

እኔወላጅ/አሳዳጊ,

በፈቃደኛነትበጥናትተሳታፊየሆንኩትየተሳታፊነትማሳወቂያቅፁንከመፈረሜአስቀድሞሥለጥ ናቱአካሄድእናየጥናቱተሳታፊመብቶችግልፅበሆነመልኩከመረጃቅፁአንብቤተረድቻለሁ /ተነቦልኝበግልፅተብራርቶልኛል፡፡

የተሳታፊስም፡- _____የተሳታፊቤተሰብስም፡- _____

_____የተሳታፊቤተሰብፊርማ፡- ቀን፡-_____

የገለልተኛምስክርስም፡- _____

የገለልተኛምስክርፊርማ፡-____ቀን___

የተመራማሪው/ዋሰም፡-_____ፊርማ፡-_____ቀን፡-

ለወላጅ/አሳዳጊየስምምነትመግለጫቅጽዕድሜያቸውከ12-17 ለሆኑተሳታፊዎች

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□□□/□□□□

የጥናቱርእስ፡- Therapeutic efficacy of Artemether-Lumefantrine (AL) plus Single Dose Primaquine (PQ) for the treatment of uncomplicated *Plasmodium falciparum.*

እኔወላጅ/አሳዳጊልጄበፈቃደኛነትበጥናትተሳታፊእንዲሆንየፈቀድኩትየተሳታፊነትማሳወቂያቅ ፁንከመፈረሜአስቀድሞሥለጥናቱአካሄድእናየጥናቱተሳታፊመብቶችግልፅበሆነመልኩከመረ ጃቅፁአንብቤተረድቻለሁ /ተነቦልኝበግልፅተብራርቶልኛል፡፡

የተሳታፊስም፡- _____

_____የተሳታፊቤተሰብስም፡- _____

_____የተሳታፊቤተሰብፊርማ፡-_____ቀን፡-_____

_____የገለልተኛምስክርስም፡-_____

_____የገለልተኛምስክርፊርማ፡-_____ቀን_____

የተመራማሪው/ዋሰም፡-_____ፊርማ፡-_____ቀን፡-____

□□□□□□□□□□□□ □□□□□□□□□□□

□□□/□□□□

የጥናቱተሳታፊየስምምነትመግለጫቅጽዕድሜያቸውከ 18 and aboveዓመትለሆኑ

የጥናቱርእስ፡ *In-vivo* Therapeutic efficacy of Artemether-Lumefantrine (AL)plusSingle DosePrimaquine (PQ) forthetreatmentofuncomplicated *Plasmodiumfalciparum*in a co-endemic setting

(Bambasi)’’እኔበፈቃደኛነትበጥናትተሳታፊየሆንኩትየተሳታፊነትማሳወቂያቅፁንከመፈረሜአ ስቀድሞሥለጥናቱአካሄድእናየጥናቱተሳታፊመብቶችግልፅበሆነመልኩከመረጃቅፁአንብቤተረ ድቻለሁ

/ተነቦልኝበግልፅተብራርቶልኛል፡፡

መፃፍለማይችሉየጣት አሻራአዚህያሳርፉ ወላጅ/ያሳዳጊ

የተሳታፊስም፡- _____

የተሳታፊፊርማ፡--------ቀን፡---------

የገለልተኛ ምስክር ስም፡-_____

የገለልተኛምስክርፊርማ፡- _____ቀን፡- ____

የተመራማሪው/ዋሰም፡- _____

የተመራማሪው/ዋፊርማ፡-____ቀን፡- _____

የጥናቱተሳታፊየስምምነትመግለጫቅጽዕድሜያቸውከ18 ዓመትበላይለሆኑተሳታፊዎችየጥናቱርእስ፡Ethiopia*in-vivo* efficacystudy2019:TherapeuticefficacyofArtemether-Lumefantrine (AL)plusSingleDosePrimaquine(PQ)forthetreatmentofuncomplicated*Plasmodiumfalcipa rum* andChloroquine(CQ)plus14daysPQforuncomplicated*Plasmodiumvivax*

እኔበፈቃደኛነትበጥናትተሳታፊየሆንኩትየተሳታፊነትማሳወቂያቅፁንከመፈረሜአስቀድሞሥለ ጥናቱአካሄድእናየጥናቱተሳታፊመብቶችግልፅበሆነመልኩከመረጃቅፁአንብቤተረድቻለሁ

/ተነቦልኝበግልፅተብራርቶልኛል፡፡

□□□□□□□□□□□□□□□□□□□□□□

የተሳታፊስም፡- _____የተሳታፊፊርማ፡-_____ቀን፡-_____

የገለልተኛምስክርስም፡- _____

የገለልተኛምስክርፊርማ፡- _____ቀን፡- _____

የተመራማሪው/ዋሰም፡- _____

የተመራማሪው/ዋፊርማ፡-____ቀን፡- _____