# Comparison of the effect of patients' own sera versus naïve sera on infectivity of *Plasmodium vivax* to *Anopheles arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia

BY: WAKWEYA CHALI GERBA

A Thesis Submitted to the Department of Applied Biology

Presented in Partial Fulfillment for the Requirements of the Degree of

Master of Science in Applied Biology (Biotechnology)

School of Applied Natural Science

Office of Graduate Studies

Adama Science and Technology University

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School of Applied Natural Science

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Adama, Ethiopia

# Declaration

I hereby declare that this MSc thesis entitled "Comparison of the effect of patients' own sera versus naïve sera on infectivity of *Plasmodium vivax* to *Anopheles arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in 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 that are used for this thesis have been duly acknowledged through citation.

| Wakweya Chali Gerba | _____ | _____ |
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| Name of the student | Signature | Date |

## Recommendation

I/we, the advisor(s) of this thesis, hereby certify that I/we have read the revised version of the thesis entitled "Comparison of the effect of patients' own sera versus naïve sera on infectivity of *Plasmodium vivax* to *Anopheles arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia" prepared under my/our guidance by Wakweya Chali submitted in partial fulfillment of the requirements for the degree of Mater's of Science in Applied Biology (Biotechnology).

Therefore, I/we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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| Professor Hunduma Dinka | _____ | _____ |
| Major Advisor | Signature | Date |
| Dr. Fitsum Girma | _____ | _____ |
| Co-advisor | Signature | Date |

# APPROVAL SHEET

I/we, the advisors of the thesis entitled "Comparison of the effect of patients' own sera versus naïve sera on infectivity of *Plasmodium vivax* to *Anopheles arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia" and developed by Wakweya Chali, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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| Professor Hunduma Dinka | _____ | _____ |
| Major Advisor | Signature | Date |
| Dr. Fitsum Girma | _____ | _____ |
| Co-advisor | Signature | Date |

We, the undersigned, members of the Board of Examiners of the thesis by Wakweya Chali have read and evaluated the thesis entitled "Comparison of the effect of patients' own sera versus naïve sera on infectivity of *Plasmodium vivax* to *Anopheles arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in 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 Applied Biology (Biotechnology).

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# LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **AHRI** | Armauer Hansen Research Institute |
| **DBS** | Dried Blood spot |
| **DNA** | Deoxyribonucleic Acid |
| **giRBC** | Gametocyte infected Red blood cell |
| **KAHRP** | Knob Associated Histidine Rich Protein |
| **PBS** | Phosphate Buffer Saline |
| **pfEMP** | *P.falciparum* Erythrocyte membrane protein |
| **pfGEXP** | *P.falciparum* Gametocyte Exported protein |
| **qPCR** | Quantitative Polymerase Chain Reaction |
| **RBC** | Red Blood cell |
| **RNA** | Ribonucleic Acid |
| **TBV** | Transmission blocking vaccine |
| **TRI** | Transmission Reducing Immunity |
| **WBC** | White Blood cell |
| **WHO** | World Health organization |

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# ABSTRACT

*Despite the achievements obtained so far, malaria continued to be the burden of public health which may indicate the need to update and strengthen the malaria control strategies. One potential candidate that the strategy toolbox can benefit from is malaria transmission blocking/reducing vaccines. In mosquito membrane feeding experiments, where mosquitoes were artificially fed on the blood of infected humans, they could not develop oocysts showing that some individuals produced antibodies against the sexual stage surface antigens of the parasite in blood which can be taken by mosquitoes during blood meal. This study focused on comparing the effect of these sexual stage immunity in the patients' own serum with naïve serum as transmission reducing immunity from naturally P. vivax infected gametocyte carriers using serum replacement direct membrane feeding assay. The effect of patients' own sera on infectivity of P. vivax and mixed infection (P. vivax and P. falciparum) to laboratory reared Anopheles arabiensis from naturally infected patients around Arbaminch town, Ethiopia, was assessed by comparing with naïve serum using serum replacement membrane feeding assay. Feeding assays used 4–6 days-old female Anopheles arabiensis mosquitoes after starvation for 12 hrs. Oocysts development was assessed microscopically seven days post-feeding. Asexual parasites and gametocytes were quantified in donor blood by microscopy and quantitative PCR (qPCR / RT-qPCR). Eighteen individual participants confirmed of malaria positive included in feeding experiments of which 11/18 (61.1%) were P. vivax mono infection, 1/18 (5.5%) was P. falciparum mono infection and 3/18 (16.7%) were mixed infection whereas 3/18 (16.7%) of them later confirmed by nested and qPCR to be negative. A total of 3600 Anopheles arabiensis used for feeding from which >80% successfully fed. For each individual feeding, midgut of 30-32 Anopheles mosquitoes were dissected to detect and count the oocysts number per midgut. The proportion of infected mosquitoes for patients' own and naive serum was not significantly associated with gametocyte density determined by microscope ($\rho_{(11)}$ = -0.0275; P = 0.946) and stage based transcript RT-qPCR ($\rho_{(11)}$ = 0.477; P = 0.138). Likewise, it is not also associated with parasite density assessed by microscopy ($\rho_{(11)}$ = -0.0459; P = 0.452) and qPCR ($\rho_{(11)}$ = -0092; P = 0.989). From total feeding done, 83.3% (10/12) of P. vivax single species and 33%, (1/3) of mixed species patients infected at least one mosquito whereas no mosquitoes infected by P. falciparum. The proportion of infected mosquitoes increases*

*when naive serum (median of proportion of infected mosquitoes, 99%; IQR, 67-100) than the patient's own serum (median 80%; IQR, 43-87; P=0.0023, Wilcoxon signed-rank test) was used. The overall median oocysts intensity per infected midgut was significantly lower in patient's own sera (median, 10; IQR, 3-30; range, 1-187; P<0.001; Signed-rank test) than naive serum (median, 38; IQR, 6-74; range, 1-224). Transmission reduction characteristics of individual patient serum was measured by a reduction in the oocysts number and classified as 18.2% non-blocking sera and 81.8% reducing sera. There is an indication that there are antibodies from naturally infected individuals in malaria exposed populations in Ethiopia that act as a transmission reducing immunity in our study. This need further study on such antibodies for their prevalence, robustness, kinetics and blocking, or reducing, or enhancing potential against selected antigens in different endemic settings*

**Key words:** Gametocytes, immunity, membrane feeding, serum replacement, transmission

# 1. INTRODUCTION

## 1.1. BACKGROUND

The globally concerted and intensified malaria control efforts of the last decade resulted in a dramatic decline of burden of malaria on the public health. As a result, between the years 2010 and 2018, morbidity declined from 251, 000 to 228, 000 cases and mortality reduced from 585,000 to 405, 000 deaths worldwide (WHO, 2019). Despite these achievements though, malaria continued to be the burden of public health which may indicate the need to update and strengthen the malaria control toolbox strategies. One potential candidate that the toolbox can benefit from is malaria transmission blocking/reducing vaccines.

Malaria is caused by infection with protozoan parasites belonging to the genus Plasmodium, transmitted by female Anopheles mosquitoes (Cox, 2010). Human malaria is predominantly caused by four Plasmodium species: *P. falciparum, P. vivax, P. ovale* and *P. malariae.* Although there are limited evidences for zoonotic transmission, *P. knowlesi* was considered to be the fifth (Lee *et al.*, 2009; Singh *et al.*, 2004). *P. falciparum* and *P. vivax* are the most prevalent agents of human malaria.

*Plasmodium* parasites have a complex life cycle that alternates between a vertebrate host and a mosquito vector. Even though Plasmodium parasites have a rather restricted vertebrate host range, they have adapted to at least 70 different mosquito species (Sinka *et al.*, 2012). Gametocytes are the only *Plasmodium* life-stages that are infectious to mosquitoes, so that the uptake of these specialized forms during the blood meal of female Anopheles mosquitoes is essential for transmission. For *P. falciparum*, gametocyte formation is a 10-12-day process, the longest of all *Plasmodium* sp., during which the parasite passes through five morphologically distinct forms. *P. falciparum* gametocytes are formed when asexual schizonts become committed to producing sexual progeny by the activation and expression of Apatella2-g (*AP2-G*) (Kafsack *et al.*, 2014; Sinha *et al.*, 2014) the master transcriptional regulator of gametocytogenesis that is under the control of its upstream regulator nuclear protein gametocyte development 1 (Filarsky *et al.*, 2018). Immature gametocytes (stages I-IV) sequester outside the peripheral circulation, primarily to the bone marrow (Joice *et al.*, 2014), and are released after full maturation (Aguilar *et al.*, 2014). The

mature stage V gametocytes then become accessible in the peripheral blood for uptake by blood feeding mosquitoes (Pelle *et al.*, 2015).

The development of *P. vivax* gametocytes is markedly faster than *P. falciparum*, also involves a bone-marrow phase (Haiyambo *et al.*, 2019) and requires only ~48 hours for maturation (Sinden R, 2002). The circulation time of *P. falciparum* and *P. vivax* gametocytes also differs significantly. Whilst mature *P. falciparum* gametocytes can be detected for several weeks after clearance of asexual parasites (Dicko *et al.*, 2018; Reuling *et al.*, 2018), *P. vivax* gametocyte half-life is very short with microscopically detectable gametocytes and gametocyte-specific mRNA disappearing within days of asexual clearance (McCarthy *et al.*, 2013; McKenzie *et al.*, 2002a). Stage V *P. falciparum* gametocytes can be recognized in blood films by their characteristic crescent shape. In contrast, mature *P. vivax* gametocytes display a round shape and fill almost the entire red blood cell (Sinden, 2002).

Many factors appear to influence the likelihood of gametocytes being transmitted to mosquitoes and establishing a successful mosquito-stage infection (T. Bousema *et al.*, 2011). This could include parasite (sex ratio and duration of infection), host (immunity and anemia) and environmental (endemicity and season) factors. Antibodies derived from host during blood meal are also highly responsible for complement-mediated killing of gametocytes (sexual stages) and prevent gamete fusion in mosquito (Dennison *et al.*, 2015).

Even though transmission immunity is the most studied and an influential factor determining infectivity of malaria patients (Gamage-Mendis *et al.*, 1992) there are few reports on the ability of antibodies in killing gametocyte through complement-mediated lysis and preventing sequestration and maturation of gametocytes in the host (Dennison *et al.*, 2015). Exposure to the sexual stages of Plasmodium, or to specific sexual-stage antigens, can induce transmission-reducing immunity (TRI), immunity elicited in the blood, which functions only in the mosquito (Gamage-Mendis *et al.*, 1992). Therefore, this study focused on comparing the effect of sexual stage immunity as transmission reducing immunity from naturally infected gametocyte carriers using serum replacement direct membrane feeding assay.

## 1.2. STATEMENT OF THE PROBLEM

The infectivity of gametocytes to mosquitoes is influenced by numerous factors, including gametocyte density (Mendis *et al.*, 1981), maturity (Chansamut *et al.*, 2012), sex ratio (Tadesse *et al.*, 2018a), and human immune factors (Stone *et al.*, 2016). Human immunity may influence gametocyte transmission either by affecting gametocyte formation and survival in the blood, or by affecting the life stages that emerge after ingestion by mosquitoes. There is some evidence that inflammatory cytokines (TNF-a) induce cell-mediated killing of asexual parasites and gametocytes in hosts experiencing acute paroxysm (Naotunne *et al.*, 1993).

Additionally, gametocytes that are not transmitted to mosquitoes get degraded in the blood, eliciting immune responses against gametocyte antigens that are inaccessible to antibodies while gametocytes are circulating intact in the blood stream. These gametocyte-specific antibodies may be ingested by mosquitoes alongside transmissible gametocytes, and if these antibodies interact with parasite proteins involved in gametocyte activation or gamete fertilization they may inhibit the parasites' further development in the mosquito. In this way, exposure to the sexual stages of *Plasmodium*, or to specific sexual-stage antigens, can induce transmission-reducing immunity (TRI), immunity elicited in the blood, which functions only in the mosquito (Gamage-Mendis *et al.*, 1992).

Even though extensive studies in some African countries on this transmission showed reduction of immunity that will help in the development of transmission blocking vaccines that are important in malaria control and elimination, there is no study conducted under Ethiopian situation. So, extensive study on this transmission reducing immunity will help in the development of transmission blocking vaccines that are important in malaria control and elimination.

## 1.3. SIGNIFICANCE OF THE STUDY

Malaria remains one of the most important infectious diseases, causing high morbidity and mortality especially in Sub-Saharan Africa. Major efforts to reduce the burden of malaria have been undertaken in the last two decades, with considerable reductions in clinical incidence being attributed to improved access to efficacious treatment and vector control (von *et al.*, 2016).

One of the major challenges for malaria elimination initiatives is the efficient spread of malaria from infected humans to mosquitoes (Rabinovich *et al.*, 2017). Interventions that target this process and interrupt transmission to mosquitoes may be crucial for some areas to achieve elimination. The identification of novel gametocyte-specific targets of naturally acquired immunity against different gametocyte stages could aid in the development of potential TBV targets and ultimately an effective transmission blocking approach that is important in malaria control and elimination strategies. Thus, people at risk of malaria infection, government and private sectors working on malaria elimination and eradication strategies will benefit from this study.

## 1.4. SCOPE OF THE STUDY

The main concern of this study was to compare the effect of patient's own serum versus naïve serum on *P. vivax* mono infection and mixed infection of *P.vivax* and *P. falciparum* infectivity to locally reared *A. arabiensis* from naturally infected gametocyte carriers using serum replacement membrane feeding assay. This study was limited to Ethiopian naturally infected gametocyte carriers for *P. vivax* and major vector known for their transmission, *A. arabiensis*, to generate additional information that will help the scientific community working on transmission blocking vaccines.

## 1.5. HYPOTHESIS

**$H_o$** = Sexual stage immunity in patients' own sera have no effect on *P. vivax* and *P. falciparum* transmission from naturally infected gametocyte carriers to *A. arabiensis*

**$H_a$** =Sexual stage immunity in patients' own sera have effect on *P. vivax* and *P. falciparum* transmission from naturally infected gametocyte carriers to *A. arabiensis*

**$H_O = \mu_o = \mu$** where **$H_o$** is null hypothesis, and

**$H_a \neq \mu_o \neq \mu$, $H_a$** is alternative hypothesis to be tested

# 2. OBJECTIVES

## 2.1. General objective

- To compare the effect of patients' own sera versus naïve sera on *P. vivax* and mixed infection (*P. vivax* & *P. falciparum)* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using serum replacement membrane feeding assay in Ethiopia.

## 2.2. Specific objectives

- To compare the transmission of *P. vivax* and *P. falciparum* parasites to *A. arabiensis* between direct whole blood and serum replaced blood using direct membrane feeding assay.
- To determine the association between parasite density and the likelihood of infectivity to mosquitoes as determined by microscopy and quantitative PCR (qPCR).
- To find out the association between gametocyte density and likelihood of infectivity to mosquitoes as determined by microscopy and quantitative PCR (qPCR).

# 3. LITERATURE REVIEW

## 3.1. Epidemiology and life cycle of *Plasmodium* parasites

The majority of the malaria cases are caused by infection with *Plasmodium falciparum* or *Plasmodium vivax.* Whilst *P. falciparum* is the dominant Plasmodium species in most of Africa and is associated with the most severe morbidity and mortality, *P. vivax* is more widely distributed and is increasingly recognized as a major source of morbidity and economic retardation (Ahmed *et al.*, 2014).

Malaria transmission depends upon successful sexual differentiation and maturation of parasites in the vertebrate host and further development in the mosquito midgut. Stage-specific proteins in the sexual stages have been shown to play a critical role in development and successful transmission through the *anopheles* mosquito vector (Cao *et al.*, 2018a) (Figure 1).

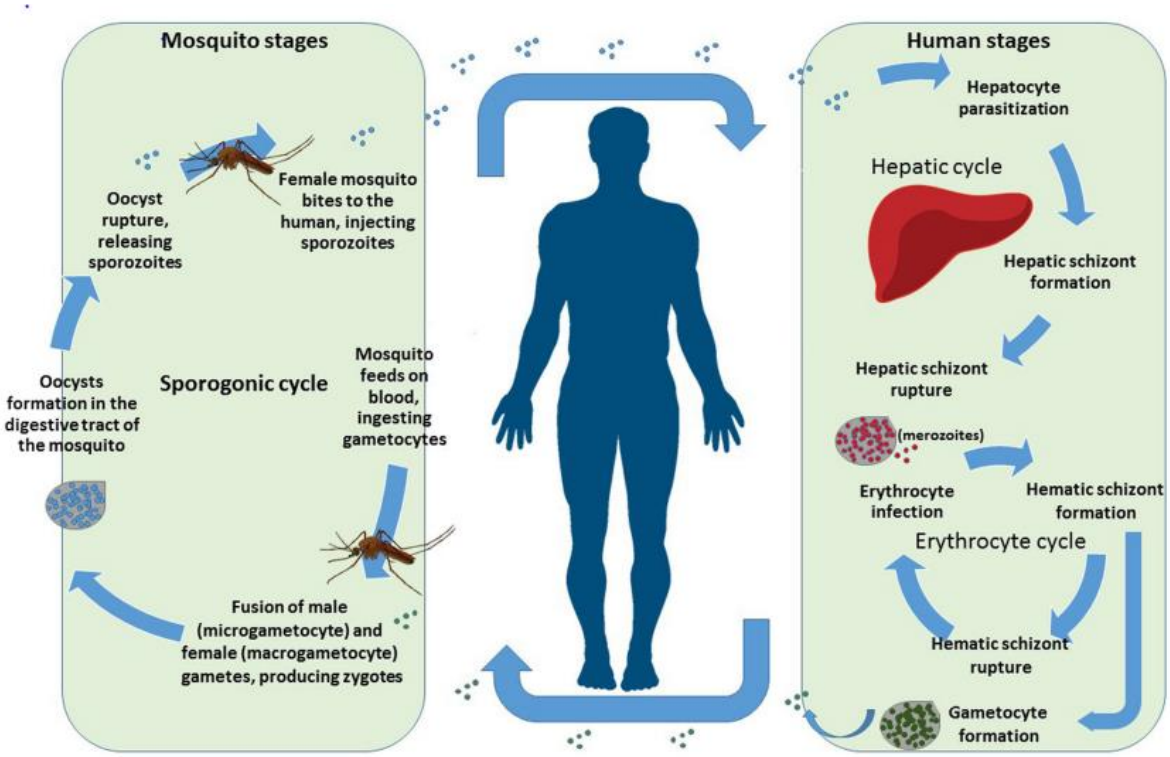


Figure 1. *Plasmodium* life cycle (Garrido-Cardenas *et al.*, 2019)

Some studies assessed the presence of antigen-specific *P. vivax* sexual stage antibodies, which have association with specific antibody responses with transmission reducing immunity (Arévalo-Herrera *et al.*, 2015). These antibodies can be found in the serum of individuals exposed to plasmodium parasite and be taken by *Anopheles* mosquitoes during blood meal and interfere with parasite development in the mosquito gut (Cao *et al.*, 2018a).

One of the unique features of *P. vivax* is the earlier generation of gametocytes, i.e. within 3–4 days after the first appearance of asexual parasites and has great association with its density (McKenzie *et al.*, 2002b). As a result, most patients start infecting mosquitoes before the onset of symptoms (Boyd *et al.*, 1937).

The emergence of resistance of Plasmodium parasites to antimalarial and resistance of mosquitoes to insecticides are major concerns when moving forward with malaria control; progress has halted in recent years and some countries are experiencing increases in malaria burden in recent years (Dondorp *et al.*, 2009). WHO estimates that there were 219 million malaria cases due to malaria in the year 2017. This figure has remained relatively static since 2015 (WHO, 2019), indicating that recent malaria control strategies have not been successful in reducing global transmission rates.

Immature and mature gametocytes have markedly different biology, morphology, and preferential localization in human tissues. Immune responses to early and late gametocytes affect transmission potential differently; early gametocyte immunity has the potential to reduce the number of gametocytes achieving maturity in the peripheral blood, while late gametocyte immunity may affect gametocyte number and their likelihood of undergoing successful sporogonic development in the mosquito (Huff *et al.*, 1958).

## 3.2. Cell mediated immunity

After repeated exposure to malaria, individuals eventually develop effective immunity that controls parasitemia and prevents severe and life-threatening complications (Marsh *et al.*, 2006). Antibodies are a crucial component of naturally acquired protective immunity against blood stage malaria with roles that may include inhibition of merozoite invasion into new red blood cells (RBCs), blocking cytoadherence of infected RBCs (iRBCs) to endothelial cells, and enhancing

phagocytic activity of monocytes and macrophages (Beeson *et al.*, 2008; Wipasa *et al.*, 2002). Effective immunity seems to require both humoral and cellular immune responses, probably in co-operation, although the relative importance of each remains unclear. The acquired response is thought to target predominantly blood-stage parasites, but antigens expressed by sporozoites and malaria-infected hepatocytes also seem to be important (Beeson *et al.*, 2008).

Our understanding of cell-mediated immunity (CMI) in malaria remains relatively poor, despite the recognition that CD4+ T-cell help is essential for most *Plasmodium* specific antibody responses (Good *et al.*, 2005), and evidence that vaccine induced *P. falciparum*-specific CD4+ T-cell responses might protect against malaria in humans (Pombo *et al.*, 2002).

A large body of research on interactions between *Plasmodium* pre-erythrocytic stages and the host has also identified important roles for memory CD8+ T cells in protection from re-infection, again thought to depend on CD4+ T-cell help (Shedlock *et al.*, 2003). Thus, CMI responses have crucial roles in protective immunity, but also have the potential to cause tissue pathology and contribute to the development of severe malaria (Clark *et al.*, 2008). Hence, CMI regulation is important for determining whether host immune responses can effectively control parasite growth or whether they contribute to disease (Clark *et al.*, 2008).

The role of cellular immune mechanisms in the clearance of circulating gametocytes is contentious. Phagocytosis of merozoites and erythrocytic blood stages is well documented. Phagocytosis of early gametocytes occurs (Smith *et al.*, 2003), but unlikely to have a major role in reducing gametocyte density. Phagocytosis of mature gametocytes appears not to occur – possibly due to the loss of surface antigens shared by erythrocytic asexual (eg PfEMP1). In the mosquito, phagocytosis is probably inhibited by the temperature, acidity, etc (J Healer *et al.*, 1999a). For *P. vivax*, it has been shown that innate immunity and the subsequently induced cellular immune response could be halted by phagocyte inactivation (Fernandez-Arias *et al.*, 2013; Vallejo *et al.*, 2018)

### 3.2.1. Naturally acquired immunity to early gametocyte antigens

Both the asexual and sexual intra-erythrocytic forms of *P. falciparum* sequester to avoid prolonged circulation in the blood. Asexual parasites sequester in the tissue microvasculature through well-

defined ligand-receptor interactions; Knob-associated histidine rich protein (KAHRP) is critical to the formation of 'knobs' on the infected erythrocyte surface (Rug *et al.*, 2006), while members of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family accumulate on these knobs and mediate cytoadherance (Kraemer *et al.*, 2006). The immature developmental forms of *P. falciparum* gametocytes sequester primarily in the vascular niche of the bone marrow and spleen (Aguilar *et al.*, 2014; Farfour *et al.*, 2012; Joice *et al.*, 2014; Smalley *et al.*, 1981). Prior to their sequestration, gametocyte development is marked by changes in gene expression. *P. falciparum* gametocyte exported protein 5 (PfGEXP5) is detectable from 14h after RBC invasion by a sexually committed merozoite (Tibúrcio *et al.*, 2015), and Pfs16 and Pfg27 are detectable from 24-30h post invasion (Baker *et al.*, 1994; Sharma *et al.*, 2003). At the same time, proteins associated with asexual stage cytoadherence are down regulated.

By stage II, gametocytes are morphologically distinguishable from asexual trophozoites, knobs are no longer visible on their surface, KAHRP protein is undetectable, and residual PfEMP1 protein is present only at low levels (Rug *et al.*, 2006). The role of PfEMP1 in early giRBC adhesion to the bone marrow vasculature is unclear. Adherence of giRBC to either C32 melanoma cells or human bone marrow endothelial cells has been demonstrated (Rogers *et al.*, 1996; Rogers *et al.*, 2000), but a later study did not detect any adherence of gametocyte stages later than I and IIa to C32 cells (Day *et al.*, 1998), and Tibúrcio et al., (2013) demonstrated only limited adhesion to a variety of endothelial cells. Recently, adhesion experiments using bone marrow mesenchymal stromal cells demonstrated that immature gametocytes were able to adhere to this cell type via unknown ligands on the giRBC surface (Messina *et al.*, 2018). These data provide evidence for the presence of surface molecules on immature giRBC that are involved in adhesion. These parasite ligands are potentially immunogenic targets (Tibúrcio *et al.*, 2013).

There has been some evidence reported indicating that naturally induced antibodies in sera from malaria patients affect the morphology and infectivity of gametocytes by binding to immature forms from stage II onwards (Tonwong *et al.*, 2012). Immune responses against antigens on the immature giRBC could affect gametocyte development or circulation time by interfering with sequestration or mediate direct clearance. An epidemiological study performed in Indonesia in the early 1990s demonstrated that gametocytes could be repressed by gametocyte specific humoral

immune responses independent of responses against asexual parasites (Baird *et al.*, 1991). These data gave rise to the hypothesis that naturally acquired antibodies against surface antigens on giRBC may directly affect gametocyte densities in circulation independent of factors reducing the asexual parasite biomass.

Several studies aimed to identify the erythrocyte surface antigens of immature gametocytes that could be involved in sequestration and efforts need to be directed toward looking at antigens on the surface of sexually committed merozoites, and schizonts containing sexually committed merozoites. Contrary to the expectations of the authors, immune sera from Gambian and Ghanaian gametocyte carriers only showed reactivity with the surface of early and mature giRBC from both 3D7 as well as clinical isolates (Dinko *et al.*, 2016; Saeed *et al.*, 2008).

Interestingly, a recent study demonstrated that surface recognition by naturally acquired antibodies was only present on erythrocytes infected with immature forms (I to III). In that study, no measurable reactivity with the surface of giRBC infected with mature stage V gametocytes was observed (Dantzler *et al.*, 2019). The authors emphasized that their contrasting findings could be due to the more-strict conditions used to obtain the different developmental gametocytes forms, with which red blood cell integrity and the activation of mature gametocytes into gametes were controlled for by counterstaining with antibodies specific to proteins on the gametocyte (not giRBC) surface. The authors used a transgenic parasite line and flow cytometry to demonstrate the reactivity of Malawian immune sera to antigens on the surface of immature giRBC, which are mostly shared with asexual infected erythrocytes. Subsequently, they used three complementary approaches to identify the targets. Firstly, they probed giRBC membranes (stage I to III) with Malawian immune plasma to identify differential protein bands between surface-intact and surface-depleted samples by mass-spectrometry. Additionally, they probed sera of mice immunized with the membranes used for mass-spectrometry on a protein microarray consisting of gametocyte proteins (Stone *et al.*, 2018). Lastly, they used the same protein microarray to constitute an immune profile of a selection of Malawian plasma samples that showed a range of membrane reactivity as identified by flow-cytometry. Combining the data of these three approaches and an initial filtering resulted in an overlapping list of 30 proteins of which 26 are predicted to be exported. The vast majority of these hits were shared with the asexual life stages.

giRBC responses were associated with increased phagocytosis of infected erythrocytes with both asexual and gametocytes. This suggests a possible mechanism of increased clearance of giRBC by antibody-mediated phagocytosis (Stone *et al.*, 2018).

Despite the remarkable differences in gametocyte development between *P. falciparum* and *P. vivax*, it has been demonstrated that there are similarities in sexual stage gene expression dynamics (Obaldia *et al.*, 2018a). This would suggest conservation of pathways involved in sexual development. In the same study the authors demonstrated that early *P. vivax* gametocytes indeed also sequester in the parenchyma of the bone marrow. There have been no reports on immune responses against immature *P. vivax* iRBCs, but given these similarities, there could be a similar phenomenon. Some limited studies on rodent models are just as likely to apply for *P. vivax* as *P. falciparum* (De Niz *et al.*, 2018; Franke-Fayard *et al.*, 2010), and clear demonstration of bone marrow sequestration by *P.vivax* (Obaldia *et al.*, 2018b).

### 3.2.2. Naturally acquired immunity to late (mature) gametocyte and gamete antigens

Despite the lack of consensus in prior studies, the progressive loss of giRBC surface recognition during gametocyte maturation (Dantzler *et al.*, 2019) suggests that antigens present on the surface of giRBC may be largely absent prior to gametocyte maturity. Mature *P. falciparum* gametocytes must circulate for long periods to maximize possibilities for mosquito transmission, and they appear well adapted to this niche. They are metabolically quiescent, accounting for their resistance to schizonticidal compounds, but primed for rapid endo-mitotic replication after activation in the mosquito midgut. Their lack of host cell surface antigens is a plausible as a mechanism of immune evasion, but the intra-erythrocytic gametocyte has already produced proteins required for RBC regress, flagellation center formation, and gamete fusion. Mature giRBCs must also pass through the sub-dermal capillaries in order to be transmitted to blood-feeding mosquitoes (Sandeu *et al.*, 2017).

Evidence that gametocytes accumulate preferentially in capillary beds is lacking (Sandeu *et al.*, 2017), however one hypothesis is that mature giRBC surface antigens bind these tissues specifically (‘tropins’), or time their release from sequestration or visceral circulation to optimize

the likelihood of ingestion by mosquitoes ('circadins') (Sutherland, 2009). STEVORs, RIFINS, and SURFINs are all hypothetical mature giRBC surface antigens, but none have been shown to mediate gametocyte accumulation in the capillaries. Gametocyte longevity appears to decrease with age, which could reflect the acquisition of age-dependent gametocyte specific humoral immunity, or could be due to active responses against all circulating Plasmodium life stages (Sutherland, 2009).

In numerous systems, the period of infection 'crisis' following peak parasitaemia has been observed to coincide with a loss of gametocyte infectivity. As erythrocytes lack MHC, direct targeting of giRBCs by T cells is not possible. However, gametocytes can be killed by reactive oxygen species induced by the indirect activation of CD4+ T cells. Serum factors from splenectomised macaques (infected with *P. cynomolgi*) taken at the point of infection 'crisis' or paroxysm can kill gametocytes, and this appears to be mediated by inflammatory cytokines including tumor necrosis factor-α (TNF α) which stimulate WBCs to produce toxic nitric oxides (Naotunne *et al.*, 1991).

In semi-immune *P. vivax* infected humans, cytokine concentrations were insufficient to induce killing factors during paroxysm (Karunaweera *et al.*, 1992) and it is unclear how this may differ in non-immune humans. Interestingly, gametocyte killing factors appear non-specific to species or parasite stage; that is, supernatant from PBMCs stimulated with *P. vivax* schizont extract was able to kill *P. falciparum* gametocytes, and *vice versa* (Naotunne *et al.*, 1993). T-cell responses seem similarly non-specific to parasite stage (Goodier *et al.*, 1997). These data present an exciting avenue for whole parasite vaccine development, but it remains unclear whether infection crisis in human's leads to meaningful levels of gametocyte death (Good *et al.*, 2017).

As described, early gametocytes produce proteins with functions in host cell deformation and others that are produced in anticipation of the intensive morphological, replicative, and sexual processes occurring after successful colonization of the mosquito gut. Whilst the presence of immune responses to mature giRBCs remains unconfirmed, there is strong evidence for naturally acquired responses to antigens shared by intra-erythrocytic gametocytes and gametes. Whilst

antigens present on the surface of viable intra-erythrocytic gametocytes are inaccessible to circulating antibodies, the immune system is exposed to them when giRBCs are cleared by the spleen (Stone *et al.*, 2016).

## 3.3. Humoral immunity

The most direct evidence that antibodies are important mediators of immunity to malaria comes from passive transfer studies in which antibodies from malaria-immune adults were successfully used to treat patients with severe malaria (Cohen *et al.*, 1961). Protective antibodies are thought to target primarily merozoite surface antigens, erythrocyte invasion ligands and variant surface antigens expressed by *P. falciparum* infected erythrocytes (IEs) (Bull *et al.*, 2002). *P. falciparum* Pfs48/45 protein is present on the male and female gametocyte surface (Kocken *et al.*, 1993; Vermeulen *et al.*, 1986a), and is retained after gamete activation in the mosquito gut; whereas, *Pfs47* is the female gametocyte/gamete paralogous (van Schaijk *et al.*, 2006b). Since these proteins are highly conserved in *P. vivax*, their expression and function is likely to be similar in both species (Y. Cao *et al.*, 2018b).

The other immunogenic cell surface protein Pvs230 showed higher expression in male and female gametocytes and gametes (Gerloff *et al.*, 2005; Tachibana *et al.*, 2012). For transmission blocking vaccine development, humoral immunity can also be extended to the gamete/zygote/ookonite surface antigens such as Pvs25(Miyata *et al.*, 2010), Pvs28 (Chaurio *et al.*, 2016; Tomas *et al.*, 2001) and HAP2/GSV1 (Hapless 2/generative cell specific protein 1) (Blagborough *et al.*, 2009). These antigens do not induce natural human immune response as they are expressed only by gametes, zygotes or ookonites. However, immunization with recombinant proteins in animal models showed that these antigens can induce antibody responses which interfere with parasite development in the mosquito mid-gut (Sala *et al.*, 2018)

*P. falciparum* Pfs48/45 protein is present on the male and female gametocyte surface (Kocken *et al.*, 1993; Vermeulen *et al.*, 1986b), and is retained after gamete activation in the mosquito gut (van Dijk *et al.*, 2001); whereas, Pfs47 is the female gametocyte/gamete paralogous (van Schaijk *et al.*, 2006a). Since these proteins are highly conserved in *P. vivax*, their expression and function is likely to be similar in both species (Cao *et al.*, 2016; Cao *et al.*, 2018a).

# 4. MATERIALS AND METHODS

## 4.1. Description of Study areas

The study was conducted in Arba Minch zuria woreda, SNNP of Ethiopia. Arba Minch is located 495 kilometers away from Addis Ababa the capital city of the country. Arba Minch zuria woreda is a district surrounding Arba Minch town. The district has two lakes (Abaya and Chamo) with evergreen forest coverage. The district has a total of eleven kebeles (lowest administrative level). The major livelihood plantation of the area is banana cultivation which is based on irrigation canals from the lakes and nearby rivers. The presence of banana cultivation irrigation fields acts as major breeding sites for the mosquitoes. As a result, most of the villages of the kebelles in the area are malarious. Sille sera kebelle is the village from which the study participants came for treatment to Shecha health center during our study period and the patients treated free of charge based on the treatment guideline (Arba Minchi Zonal Health office report 2018 unpublished data) (Figure 2).

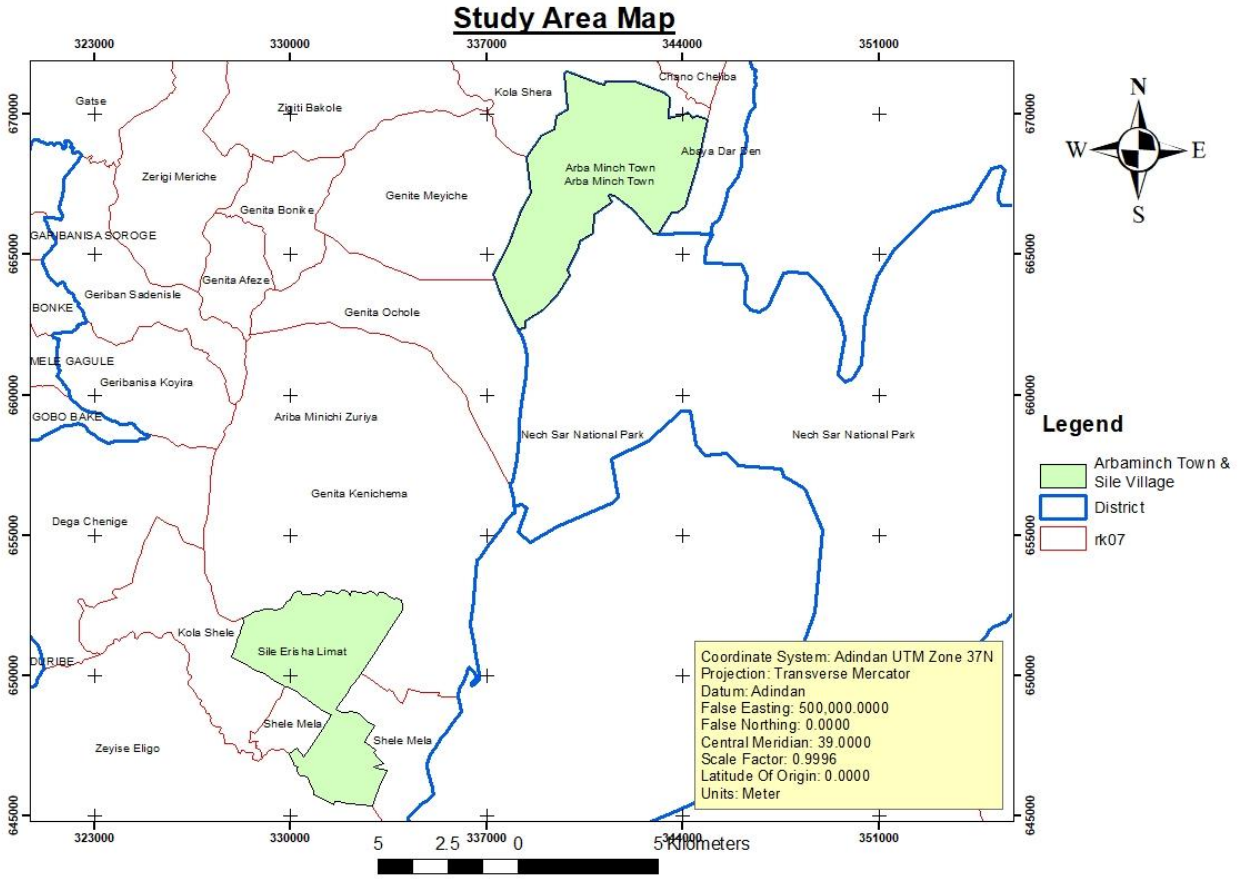


**Figure 2.** Map of study area, Arba Minch Zuria Woreda, Sille village and Arbaminch town, south-west Ethiopia (produced in ArcGIS Version: 10.8, 2020).

## 4.2. Study Population

Self-presenting individuals with microscopy confirmed *P. vivax* and mixed (*P. vivax* and *P. falciparum*) infection at health facilities were included in the study. Study participants were those above the age of 5 years requested to donate 5mL venous blood samples using syringes in to two different tubes (3mL in Serum tubes and 2mL in Heparin tubes) after explaining the objectives of the study, benefits and risks of sample collection and upon receiving signed informed written consent and/or assent (Annex I - VII) from guardians/parents for children under the age of 12 years. Eligible patients were provided appropriate treatment free of charge according to the treatment guideline of the country at the health facility.

## 4.3. Study design

Facility based cross sectional study was carried out to collect data and blood samples from self-presenting symptomatic patients at health facilities located in and around Arbaminch Zuria, confirmed of *P. vivax* and mixed (*P. vivax* and *P. falciparum*) gametocyte carriers from February 2020 – May, 2020.

## 4.4. Sample size determination and Sampling techniques

Purposive sampling was used to recurit patients with gametocyte carriers for *P. vivax* and mixed (*P. falciparum and P.vivax)* infection and volunteer to donate venous blood. The purpose and practical consequences of participating in the current study was explained to the participants. Based on the feasibility of logistics and complexity of the study during the harsh condition of COVID-19 pandemic, only available confirmed malaria cases which was 18 included in the study. But the number of female *A. arabiensis* used was determined to be 100 - 120 per individual case used for feeding and 30 *A. arabiensis* used for dissecting to detect infection from our previous studies (Chali *et al.*, 2020; Tadesse *et al.*, 2018b). These were accepted to be significant enough for analysis as number of mosquitoes used for feeding and dissection to detect infection. Therefore, a total of 18 individuals confirmed for gametocyte voluntarily donated venous blood for paired feeding. A total of 3600 *Anopheles arabiensis* were used for feeding from which >80% successfully fed. For each individual feeding, midgut of 30-32 *Anopheles arabiensis* were dissected to detect and count the oocysts number per midgut.

### 4.5. Inclusion and Exclusion criteria

#### 4.5.1. Inclusion criteria

All individuals (aged >5 years) and confirmed of gametocyte carriers were eligible to join the study once they are volunteer to participate and they gave informed written consent.

#### 4.5.2. Exclusion criteria

All individuals younger than 5 years, those with severe malaria, those who have severe anemia, pregnant women and those who didn't want to participate even if they qualify for the inclusion criteria were excluded from the study**.**

## 4.6. Data collection methods

### 4.6.1. Questionnaire

Participants and parents/guardians were requested to give information using structured interview based questionnaire (Annex-X) to capture socio-demographics, anthropometric and other relevant information. Briefly, adult participants (aged ≥18 years) or parents/guardians of children (<18 years) were asked a simple set of standardized, pre-tested questions on recent history of fever, history and nature of other malaria symptoms, history of taking anti-malarial drugs in the past month and recent travel history.

### 4.6.2. Microscopy

After finger prick, blood sample were taken from participants, so that thick and thin blood films were made, air dried, and stained with 10% Giemsa. Finally, the Giemsa stained blood films were examined under light microscope with 1000X magnification by using oil immersion and the parasite density were determined by counting the parasite against 200 WBCs and calculated as: Number of parasites counted/ 200 WBCs and multiplied by a standard average leukocyte count of 8000/µl to get parasite density per µl of blood. Both asexual stage and gametocyte densities were simultaneously assessed by counting against 200 leukocytes in the thick smear as previously described by (Ouédraogo *et al.*, 2007).

### 4.6.3. Mosquito Membrane feeding Assays

Before venous blood sample collection, all materials that were used in sample collection and membrane feeding kept at 37 °C in the incubator. Also the centrifuge was pre-warmed to 37 °C to prevent male gametocytes ex-flagellation due to temperature change before fertilization outside mosquitos' midgut.

Venous blood collected (approximately 5ml) was aliquot into serum tube (3ml) for molecular analysis and lithium heparin tubes (Vacutainer, BD) (2ml) for membrane feeding assay. Immediately, serum sample was processed (to avoid coagulation) using the 3ml blood for different analysis like, Hgb test, preparing slide for microscopy examination and parasite count, preparing DBS, transferring 100µl whole blood into 500µl RNA protect. Heparin blood was aliquot in to two each 1ml in which one aliquot kept at 37 °C until the processing for the other tube is finalized that was used in direct membrane feeding with direct whole blood. The other tube centrifuged at 3000 rpm for 5min keeping the temperature at 37 °C.

Then plasma was removed from both (with approximately 0.5ml volume) and stored in another new labeled Eppendorf tube. Then the pellet was re-suspended to equal volume with malaria-naïve serum from AB blood group and mixed well. At the last both aliquot blood was offered to colony of *A. arabiensis* mosquitoes using membrane feeding apparatus as detailed before (Teun Bousema *et al.*, 2012). Briefly, blood in heparinized tubes and serum replaced was fed to 3-5 days old female mosquitoes that were starved for ~12 hours. Mini-glass feeders (0.3ml capacity) that were covered with PARAFILM® membrane (SIGMA-ALDRICH) and connected to a water bath (at 39ºC) were used to feed 50 female mosquitoes that were kept in paper cups (Laan, Heiloo) for 25 minutes of feeding in the dark. Unfed and partially fed mosquitoes were subsequently removed 20 minutes after feeding was completed. Fully fed mosquitoes were transferred to clean custom made cages (25cmx25cmx25cm) and maintained at the rearing conditions prior to dissection for oocyst detection (days 7) at relative humidity of 60-80% and 26-28 ºC temperature with 10% sugar solution provided.

### 4.6.4. Mid gut dissection to detect Oocysts

At day 7, *P. vivax* oocysts infection was found to reach optimum growth stage from our previous experiments while *P. falciparum* oocysts showed slow growth and optimized to be dissected at

day 10 post infection (Chali *et al.*, 2020). Mosquitoes were immobilized by shaking in the paper cup and dissected under stereo microscope to remove the midgut as described previously with slight modifications (Musiime *et al.*, 2019). Briefly, the mosquito thorax was held with ultra-fine tweezers while scalpel was used to make two incisions at the second and last abdominal segment. The abdomen epithelium was gently pulled off the mosquito abdomen in a single motion. The midgut thereby remained attached to the immobilized thorax. Malpighian tubules and ovaries were removed and put aside as they might interfere with the oocysts reading if they overlap the midgut. Then the midgut was removed in 1.0% mercurochrome (SIGMA-ALDRICH), covered with clean cover slides and kept for 10 minutes to stain. Finally, it was examined under 10x light Microscope to detect the presence of oocysts and count their intensity per midgut (W. J. Stone *et al.*, 2013)

## 4.6.5.Nucleic Acid Extraction

### a. Nucleic acid extraction from DBS and RNA protect Samples

Once venous blood samples (approximately 2ml) collected in EDTA vacutainer tube, three drops of 20µl each was prepared on WhatMan No. 3 Filter paper and air dried. Dried blood spots (DBS) on filter paper were stored individually in plastic bags with silica packets and kept in dry storage cabinets and later stored at -20$^{o}$C before genomic DNA (gDNA) extraction and testing as described previously (Schwartz *et al.*, 2015).

The QIAamp DNA extraction Mini kit designed to extract DNA from DBS samples was used following the manufacturer's instruction. Cells are lysed and DNA released in a protease-containing buffer (Koontz *et al.*, 2015). Briefly, 6mm diameter DBS was cut by puncher and put in labeled tube. Then ATL buffer was added and incubated at 95$^{o}$C for 15 minutes to release the genomic content of the blood from DBS and Proteinase K (from QIAGEN) added to the solution that help in digestion of any contaminating proteins including nucleases. This prevents the DNA from nucleases enzyme attack in the solution after cell lysis. The solution containing proteinase K incubated at 56$^{o}$C for 10 minutes for optimum reaction. Then, equal volume of absolute ethanol (96-100%) added to the solution and transferred to the spin column. The spin column centrifuged at 8000rpm for 1 minute and the flow through discarded. Then released DNA is bound to a QIAamp column and purified using two wash steps with recommended buffers that remove

contaminants. Finally, 150µl DNA is released from the column in new collection tube using appropriate buffer and stored at -20ºC for downstream reactions.

Blood samples in RNA protect buffer that were collected during feeding were used for extraction of total nucleic acids using RNeasy Micro Kit from (QIAGEN) following the manufacturer's instruction. Briefly, RNA protect samples were lysed by using Buffer RLT Plus and, the lysate homogenized and well mixed by adding equal volume of 70% ethanol. The mixture was transferred to RNeasy MinElute spin column and Centrifuged. By discarding the flow-through, the RNeasy MinElute spin column was washed by RW1 buffer and RPE buffer simultaneously and 80% ethanol added to the RNeasy MinElute spin column. Finally, after centrifugation at full speed for 2 minutes, 14µl total RNA eluted in RNase-free water and stored at -20ºC for later downstream reactions.

## b. Asexual stage Parasite detection by nested PCR

Nested PCR reaction was done to investigate the presence and identification of parasite by targeting the 18S based marker. In this study 18 patients' blood samples were analyzed using a two round nested PCR to confirm the microscopy diagnosis of malaria. In the initial amplification reaction (Nest1) Gene-specific oligonucleotide primers pairs rPLU5 (5'-TTA AAA TTG TTG CAG TTA AAA CG-3') and rPLU6 (5'-CTT GTT GTT GCC TTA AAC TTC-3') was used and in the second amplification reaction (Nest 2) gene of specific parasite was amplified using species-specific primers, for *P. falciparum* (Nested 2) rFAL1 (5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3'), rFAL2 (5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3') and *p.vivax*, rVIV1 (5'-CGC TTC TAG CTT AAC CAC ATA ACT GAT AC-3'), rVIV1(5'-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3') (Snounou *et al.*, 1993). Briefly, all reactions were performed on BioRad Thermal cycler (Applied Biosystems) in 25µl total reaction volumes from 1X PCR buffer, 2mM concentration of Mgcl2, 0.25mM concentration of dNTPs, 0.25 µM concentration of each primer, 1u/reaction of Taq polymerase enzyme (from Promega) and 5µl of DNA elute. The following cycling parameters were used: 95°C for 10 minutes of denaturation and 35 cycles of 95°C for 60 s, 58°C for 60 s, 72°C for 90 s and 72°C for 10 minutes' final extension.

## c. Gel electrophoresis

Agarose gel (2%) was prepared by measuring 2g of agarose powder in 100 ml 0.5%TBE (Tris Borate EDTA) and 2µl of ethidium bromide (EtBr) (from SIGMA-ALDRICH), was mixed with gel solution before casting that was used as fluorescent dye by intercalating between DNA strands during UV visualization.

## d. Asexual stage and Gametocyte quantification by quantitative PCR

Quantitative PCR (qPCR) for parasite detection and quantification was performed by targeting the 18S rRNA small subunit gene for *P. falciparum* and *P. vivax* using primer and probe sequences described by Hermsen and Wampfler, respectively (Hermsen *et al.*, 2001; Wampfler *et al.*, 2013). The following sequences of primers and probes were used. Pf18S forward primer sequence 5'-GTA ATT GGA ATG ATA GGA ATT TAC AAG GT-3', reverse sequence 5'-TCA ACT ACG AAC GTT TTA ACT GCA AC-3' and probe sequence 6FAM-AACAATTGGAGGGCAAG–MGBNFQ and Pv18S forward primer sequence 5'-GCT TTG TAA TTG GAA TGA TGG GAA T-3', reverse sequence 5'-ATG CGC ACA AAG TCG ATA CGA AG-3' and probe sequence HEX-AGC AAC GCT TCT AGC TTA -MGB-BHQ.

All reactions were performed on Agilent real-time Thermal cycler (Agilent) using 25µl total reaction volumes, 10 µl of 2X TaqMan Fast Advanced Master Mix (Applied Biosystems), 5µl of DNA elute, forward and reverse primers at final concentrations of 833nM each, and probe concentration of 110nM. The following cycling parameters were used: 50°C for 2 minutes, 95°C for 10 min and 45 cycles of 95°C for 15 s, and 60°C for 1 minute.

For the quantification of transcription level of *P. vivax* (Pvs25) gametocytes markers, total nucleic acid was digested by DNase I prior to cDNA synthesis. Genomic human and parasite DNA was digested with the RQ1 DNase I Digest Kit following manufacturer's protocol (Promega); 8µl sample was treated in 11µl final reaction volume containing RQ1, 10x reaction buffer and stop solution (Koepfli *et al.*, 2015). All samples that passed through DNA digestion were further checked for genomic DNA contamination. At two step procedures, cDNA was first synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Bio-systems) by adding samples in a 1:1 ratio with master mix and later used as template for qPCR on the 2$^{nd}$ step. The following primers sequences were used for Pvs25, forward primer sequence, 5'-ACA CTT GTG TGC TTG ATG TAT GTC -3', reverse primer sequence, 5'-ACT TTG CCA ATA GCA CAT

GAG CAA -3', and probe sequence of 'FAM-TGC ATT GTT GAG TAC CTC TCG GAA-BHQ1'. *P. vivax*, Pvs25 transcripts were determined using TaqMan Fast Advanced Master Mix (Applied Bio-systems) using 4µl of cDNA in 24µl final reaction volume, forward and reverse primers at final concentrations of 833nM each, and probe concentration of 110nM. The following cycling parameters were used: 50°C for 2 minutes, 95°C for 10 min and 45 cycles of 95°C for 15 s, and 60°C for 1 min.

The gametocyte quantification was done based on the previously developed models (Wampfler *et al.*, 2013). Briefly, plasmid constructs from Wampfler (Wampfler *et al.*, 2013) were used to infer copy numbers by running serial dilutions ($10^6$ – $10^1$ copies/µl) of plasmids containing the respective amplicons in duplicate on each plate. In all assays of RT-qPCR, 104nM probe and 833nM primer concentrations were used. All probes were from Life technologies (Applied Bio-systems), primers were from (SIGMA ALDRICH), and CFX96™ Real-Time PCR Detection System (BIO-RAD) was used.

## 4.7. ETHICAL APPROVAL

The study was conducted under the umbrella of a bigger cohort longitudinal study that is underway in Adama and Arbaminch which was granted ethics approval from the Armauer Hansen Research Institute (Protocol number; PO32/18), Addis Ababa, Ethiopia and the National Research Ethics and Review Committee (SHE/S.M/14.4/708/19) (Annex VIII-IX). Written informed consent/assent was sought from each participant or his/her guardian in case of children for study participation (Annex I – VII**)**. Participant information sheets and consent/assent forms was made available in the local language. Parents or caretakers were asked to provide informed consent for anyone below 18 years of age.

## 4.8. DATA ANALYSIS

All analyses were performed in STATA version 13 (StataCorp., TX, USA), GraphPad Prism 5.3 (GraphPad Software Inc., CA, USA) and Microsoft excel sheet. Statistical significance was set at a P value of <0.05. The correlation between mosquito infection prevalence and gametocyte and parasite density as continuous variable was determined by Spearman's rank correlation coefficient for both patient's own serum and naïve serum. Feeding efficiency (proportion of fully-fed mosquitoes) was compared in matched experiments using the Wilcoxon matched-pairs signed-rank test. Wilcoxon signed-rank test was used to compare the proportion of infected mosquitoes between patient's own serum and naïve serum and oocysts densities per infected midgut. The bias between patients' own sera and naïve sera was compared using the Bland-Altman test. Logistic regression was performed to compare infection status between patient own serum and naïve serum using individual mosquito oocysts data. A transmission reduction characteristic of each infectious serum or individual feeding was evaluated using R-value from arithmetic mean of difference of oocysts density using log transformed R-values.

# 5. RESULTS

## 5.1. Parasite detection by Microscope and nested PCR

There were 18 individual participants confirmed of malaria positive included in feeding experiments of which 11/18 (61.1%) were *P. vivax* mono infection, 1/18 (5.5%) was *P. falciparum* mono infection and 3/18 (16.7%) were mixed infection whereas 3/18 (16.7%) found to be negative by the nested PCR, which was different from the microscope result. 1/18 (5.5%) among *P.vivax* mono infection was confirmed by qPCR than the two methods (Table 1).

Table 1. Comparison of the parasite detection by microscope, nested PCR and qPCR and their status of infectiousness between whole blood and serum replaced membrane feeding.

| Sample ID | Result of parasite detection | | | Percentage of infection | | Status of infection | |
|---|---|---|---|---|---|---|---|
| | Microscope | Nested PCR | qPCR | Whole blood | Serum replaced | Whole blood | Serum replaced |
| SR01 | Mixed* | Mixed* | Mixed* | 80 | 100 | Infectious | Infectious |
| SR02 | *P. vivax* | *P. vivax* | *P. vivax* | 93 | 100 | Infectious | Infectious |
| SR03 | *P. vivax* | *P. vivax* | *P. vivax* | 88 | 100 | Infectious | Infectious |
| SR04 | *P. vivax* | *P. vivax* | *P. vivax* | 80 | 100 | Infectious | Infectious |
| SR05 | *P. vivax* | *P. vivax* | *P. vivax* | 43 | 67 | Infectious | Infectious |
| SR06 | Mixed* | Mixed* | Mixed* | 0 | 0 | Not infectious | Not infectious |
| SR07 | *P. vivax* | Mixed* | Mixed* | 19 | 47 | Infectious | Infectious |
| SR08 | *P. vivax* | *P. vivax* | *P. vivax* | 0 | 0 | Not infectious | Not infectious |
| SR09 | *P. vivax* | *P. vivax* | *P. vivax* | 0 | 0 | Not infectious | Not infectious |
| SR10 | *P. vivax* | Negative | Negative | 0 | 0 | Not infectious | Not infectious |
| SR11 | Mixed* | Negative | Negative | 0 | 0 | Not infectious | Not infectious |
| SR12 | *P. vivax* | Negative | *P. vivax* | 53 | 93 | Infectious | Infectious |
| SR13 | *P. vivax* | *P. vivax* | *P. vivax* | 80 | 100 | Infectious | Infectious |
| SR14 | *P. vivax* | *P. vivax* | *P. vivax* | 87 | 97 | Infectious | Infectious |
| SR15 | *P. vivax* | *P. vivax* | *P. vivax* | 3 | 63 | Infectious | Infectious |
| SR16 | *P. vivax* | *P.falciparum* | *P.falciparum* | 0 | 0 | Not infectious | Not infectious |
| SR17 | *P. vivax* | *P. vivax* | *P. vivax* | 83 | 93 | Infectious | Infectious |
| SR18 | *P. vivax* | Negative | Negative | 0 | 0 | Not infectious | Not infectious |

**P.vivax* and *P.falciparum*

Then 10µl of nested (N2) PCR product was added to each lane prepared by comb and run for 60 minutes at 120V. The result was visualized by UV light (Figure 3).

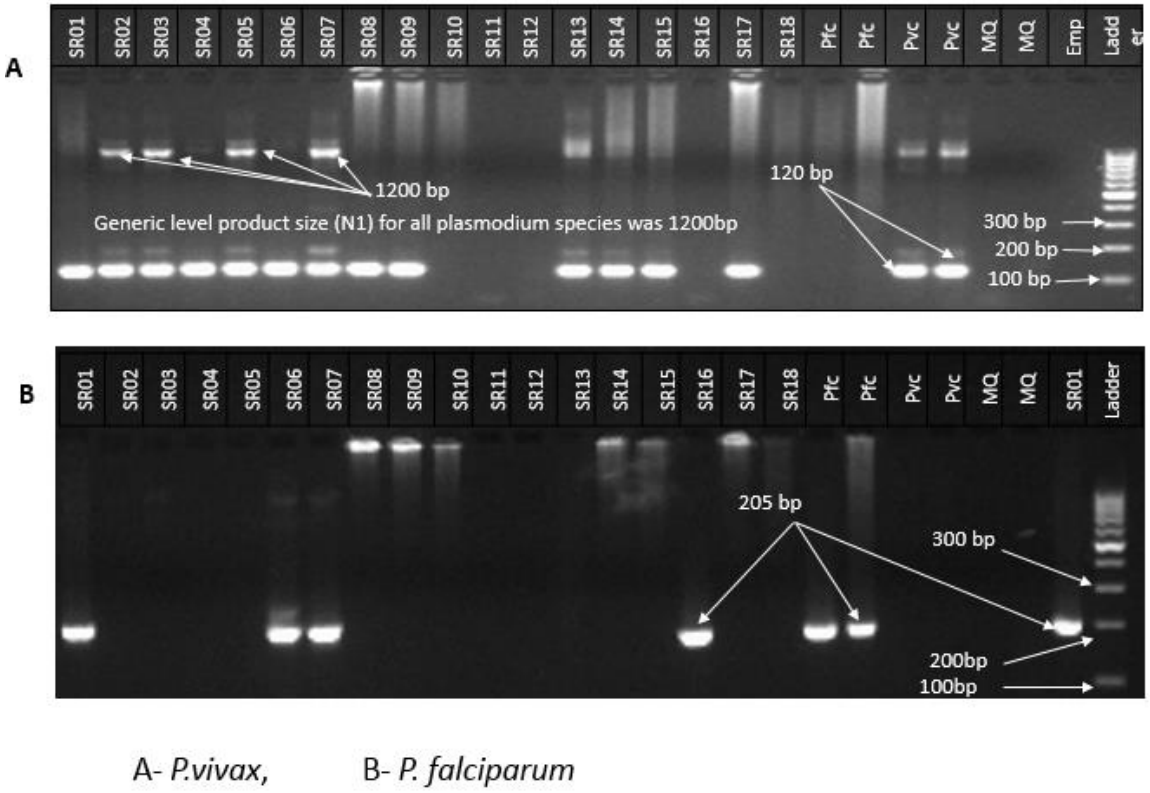


**Figure 3.** Nested PCR targeting the 18S DNA based small subunit rRNA gene. **A.** *P. vivax* gel picture at 120bp product and **B**. *P. falciparum* gel picture at 205bp product. Nested-2 PCR product for samples was added to lane 1-18, positive control added to lane 19-22 and Non template control (water) added to lane 23-24, and 1kb plus ladder added to lane 26 which was used to determine the product size at 100bp interval.

Microscopically detected parasite density was (median: 6,169; IQR: 2,971– 9,880; *n* = 15) and for that determined by 18s qPCR was (median: 11,500,000; IQR: 1,139,000 – 29,600,000; *n* = 14). Similarly, microscopically detected gametocyte density was found to be (median: 331.5; IQR: 166.5 – 1,089; *n* = 12) and for that determined by qPCR (Figure.4) using Pvs25 gametocyte marker for *P. vivax* was (median: 302,050; IQR: 49,090 - 1,346,000; n = 14) showing that qPCR was more sensitive method than Microscope.

Gametocytes were more frequently detected by microscopy in patients with *P. vivax* single-species infections (90.9%, 10/11) than in patients with *P. vivax* & *P. falciparum* co- infections (66.7%, 2/3). In co-infections, all microscopy-detected gametocytes were *P. vivax*. The proportion of infected mosquitoes for patients' own serum was not associated with gametocyte density determined by microscope ($\rho_{(11)}$ = -0.0275; $P$ = 0.946) and stage based transcript qPCR ($\rho_{(11)}$ = 0.477; $P$ = 0.138). Likewise, it is not also associated with parasite density assessed by microscopy ($\rho_{(11)}$ = -0.0459; $P$ = 0.452) and qPCR ($\rho_{(11)}$ = -0092; $P$ = 0.989). Additionally, the proportion of infected mosquitoes for naïve serum was not associated with gametocyte density determined by microscope ($\rho_{(11)}$ = 0.430; $P$ = 0.1826) and stage based transcript qPCR ($\rho_{(11)}$ = 0.11; $P$ = 0.7545). Likewise, it is not also associated with parasite density assessed by microscopy ($\rho_{(11)}$ = 0.210; $P$= 0.539) and qPCR ($\rho_{(11)}$ = -0.459; $P$ = 0.155) (Figure 3).

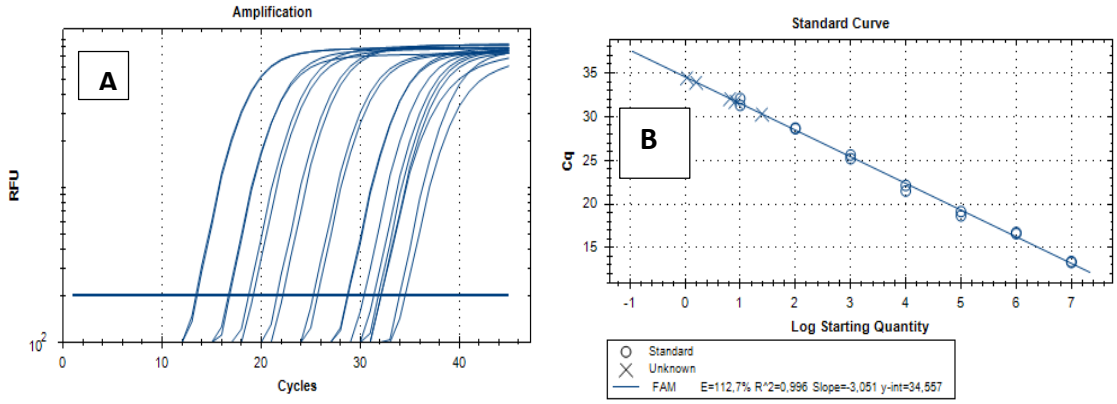


**Figure 4**. Graphical representation of quantitative PCR amplification plot (A) for *PVs25* gametocyte marker for *P.vivax* at different Cycle threshold (Ct) value and standard curve (B) for positive samples and plasmid constructs serially diluted from 10^7 - 10^1. Ct value is the minimum number of amplification cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level minimum of 500 Fluorescent dye accumulation). The minimum and maximum cut-off value for Ct value was set based on the known quantity of plasmid to 13- 40 cycle (10^7- 10^1) for the result to be significant in this experiment.

## 5.2. Comparison of infectivity and evaluation of transmission reduction between patients own and naïve serum feeding

From the total feeding experiments done, 83.3% (10/12) of *P. vivax* single species and 33% (1/3) of mixed species patients infected at least one mosquito whereas no mosquitoes infected by *P. falciparum*. Feeding efficiency was the same between patient's own serum (median proportion of blood fed mosquitoes, median, 75%; (IQR, 68-84) and naive serum (median, 78%; (IQR, 67-83) from 30-32 mosquitoes per feeding dissected for oocysts detection in midgut of blood fed mosquitoes 7 days' post feeding. In infectious feeds, the proportion of infected mosquitoes increases when naive serum (median of proportion of infected mosquitoes, 99%; IQR, 67-100) used than the patient's own serum (median 80%; IQR, 43-87; P=0.0023, Wilcoxon signed-rank test). The overall median oocysts intensity per infected midgut was significantly higher in naive serum (median, 38; IQR, 6-74; range, 1-224) than patient's own serum (median, 10; IQR, 3-30; range, 1-187; P<0.001; Signed-rank test) (Figure 5**).**

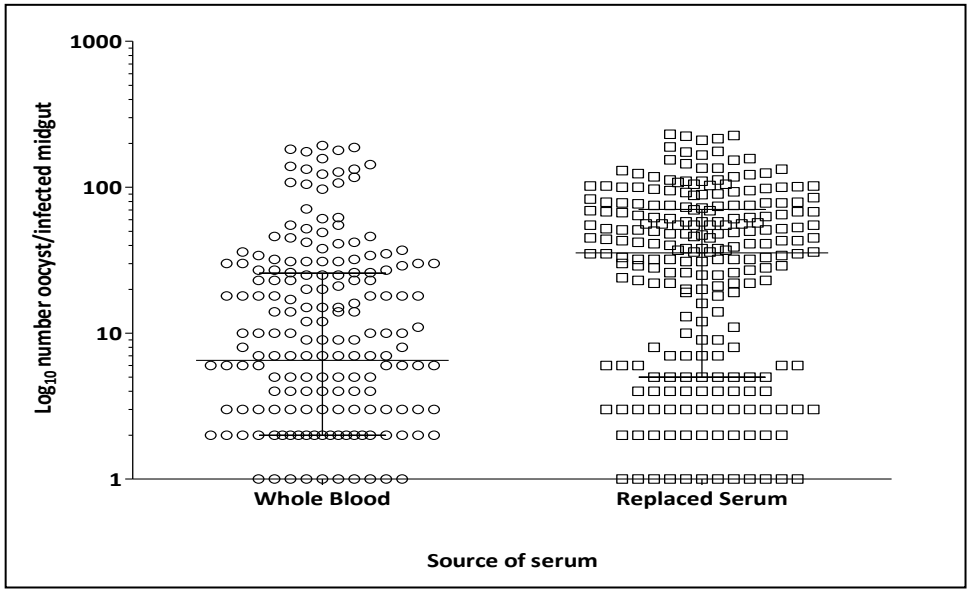


**Figure 5.** Comparison of the median of the $log_{10}$-transformed oocysts intensity per midgut of infected mosquito between whole blood patients' own serum and replaced naïve serum.

All the 11 infectious experiments showed increased mosquito infectivity when own plasma were replaced by naïve sera showing that there is antibody against sexual stages that prevent oocysts development (Figure 6.)

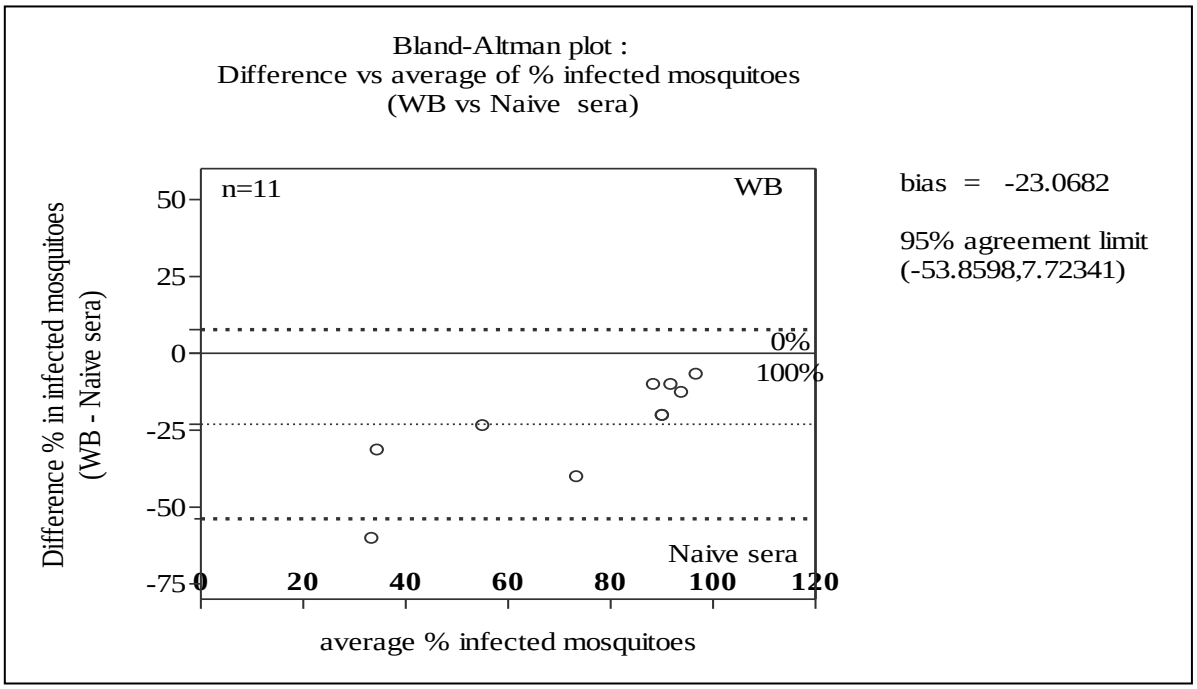


**Figure 6.** Scatter plot (Bland-Altman) for percent of infected mosquitoes of whole blood minus percent of infected mosquitoes of naïve sera (y-axis) against mean of percent in infected mosquitoes of whole blood and percent in infected mosquitoes of naïve sera. The mean difference (bias) of % infected mosquitoes was -23.07 (95% CI of -53.86 - 7.72) showing significant increase in mosquito infectivity when naïve serum were replaced by own plasma and fed to mosquitoes compared to non-replaced whole blood fed to mosquitoes.

For evaluation of transmission reduction, a reduction in the oocysts number can be measured by a so called R value, which is obtained by comparing the oocysts level resulting from use of a test serum (Twb) with that obtained with a control serum (Tnaive) (Lensen *et al.*, 1996). According to Lensen et al (1996), R values need to be calculated in order to identify blocker or non-blocker sera. R =(Tc-T)/Tc where Tc=control serum (naïve serum), T=test serum (own serum). According to this study, the usual recommended way of calculating the R value (Williamson geometric mean) can only identify blocking or non-blocking sera. However, they recommended to use arithmetic

mean on log scale of R-values, and this enables to classify sera in to three categories (R>0.9 blocking sera; 0.3<R<0.9 reducing sera; R<0.3 non-blocking sera) (Figure 7).

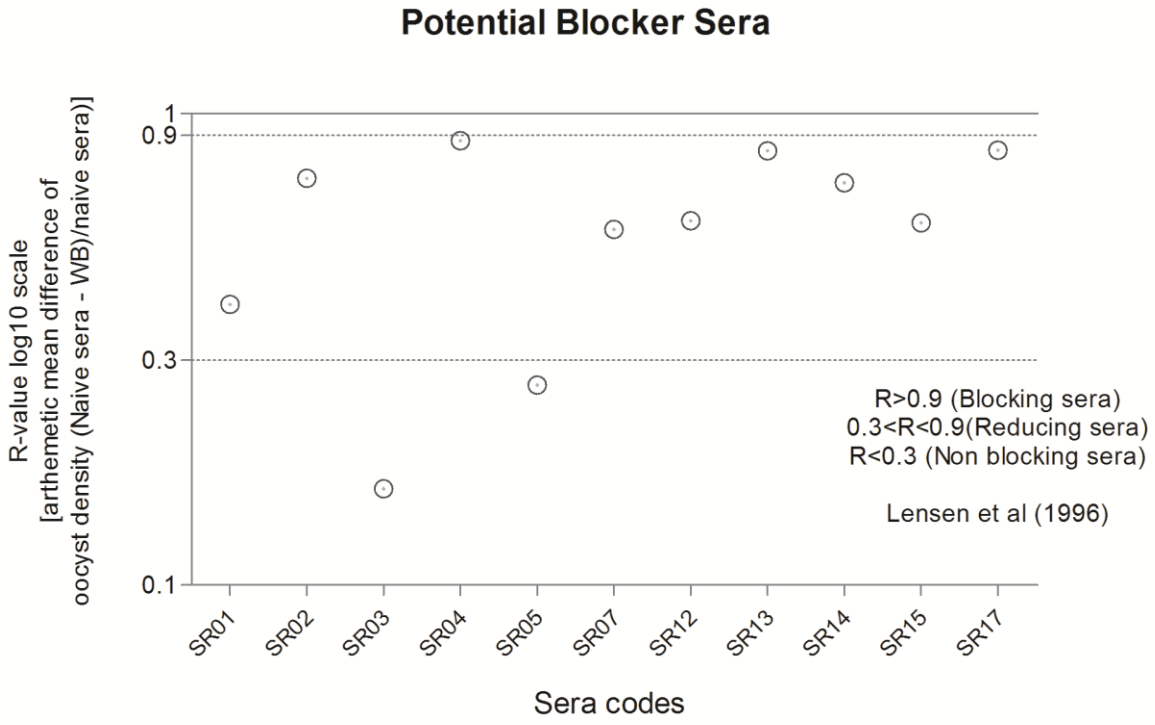


**Figure 7.** A plot for R-value from arithmetic mean of difference of oocysts density on $\log_{10}$ scale (y-axis) against individual sera (x-axis). Based on the obtained R-value, 18.2% (2/11) (SR03 and SR05) were classified as non-blocking sera, 81.8% (9/11) (SR01, SR02, SR04, SR07, SR12, SR13, SR14, SR15, and SR17) were classified as reducing sera whereas no blocking sera found from current study.

# 6. DISCUSSION

*P. vivax* malaria is commonly referred to as a major cause of malaria outside sub-Saharan Africa (Mendis *et al.*, 2001). Ethiopia forms an exception with vivax malaria, contributing towards three-quarters of the global burden together with India and Pakistan (Battle *et al.*, 2019). Even though natural transmission blocking (TB) activity for *P. falciparum* infections has been demonstrated, less is known about TB immunity in *P. vivax.* Among the factors that contribute to malaria reduction in endemic areas, TB immune mechanisms appear to play a significant role, particularly in areas of low and intermediate transmission intensity, whereas a limited impact is considered to occur in areas of high malaria transmission intensity (Arévalo-Herrera *et al.*, 2011).

Transmission-blocking activity correlated with antibody titer from the previous study conducted in Colombia, in area of low to middle malaria transmission intensity, and it was significant and apparently greater than in Mexico and Sri Lanka indicating that there is high dose of antibodies in the population from these areas comparable to our current study area with high percentage of transmission reducing sera (Arevalo-Herrera *et al.*, 2005). The fact that parasite exposure triggers an efficient antibody boosting highlights the contribution of anti-parasite immune response to produce TB. The potential of infection in boosting the antibody responses primed would be of great importance for TB vaccines that would be boosted each time the volunteer is exposed to the parasite. TB immunity is likely to be dependent on the contribution of serum complement factors and cytokines (Naotunne *et al.*, 1991) which all together may contribute to a significant reduction of malaria transmission in endemic areas and become the subject of investigation in the search for a TB malaria vaccine.

Using membrane feeding assays, abundant evidences have accumulated that malaria transmission blocking/reducing immunity exists in Plasmodium-exposed populations. This was an agreement with the present study in infectious feeds that the proportion of infected mosquitoes increased when naive serum was replaced than the patient's own serum indicating that there are important antibodies present in the serum of naturally infected individuals acting as transmission reducing activity (Bansal *et al.*, 2017). This concept came from observations where mosquitoes that were artificially fed on the blood of infected chicken (R. Carter *et al.*, 1979) and humans (K. N. Mendis *et al.*, 1987) could not develop oocysts. It was hypothesized that these chicken and individuals

could have developed immune responses which block transmission of the parasites to mosquitoes. This hypothesis was experimentally (Carter *et al.*, 1991; K. N. Mendis *et al.*, 1987)supported that some individuals can produce antibodies against the sexual stage surface antigens of the parasite in blood which can be taken by mosquitoes during blood meal; and in mosquito guts, these antibodies can halt development of zygotes and oocysts showing potential reduction in human-to-mosquito transmission (Gamage-Mendis *et al.*, 1992). Our current study also proved this hypothesis indicating that sexual stage immunity has effect on *P. vivax* transmission from naturally infected gametocyte carriers to *A. arabiensis* mosquitoes.

The most convincing data of this phenomenon was recorded related to two of gametocyte, the only transmissible forms from humans to mosquitoes (Talman *et al.*, 2004), stage antigens P48/45 and P230 (J. Healer *et al.*, 1999b; Premawansa *et al.*, 1994; Rener *et al.*, 1983) which primarily involve in male gamete fertility (Eksi *et al.*, 2006; van Dijk *et al.*, 2001). Naturally acquired antibodies against these antigens showed potential reduction in transmission of *P. falciparum* (van Dijk *et al.*, 2001) and *P. vivax* (Eksi *et al.*, 2006) gametocytes from human to mosquitoes in different endemic settings as seen during our current study which need further characterization of these specific proteins.

A limited number of studies reported a lack of association between microscopically determined gametocyte density and infectivity to mosquitoes (Sattabongkot *et al.*, 1991), which is observed in our current study that both parasite density and gametocyte density determined by microscope and molecular techniques showed no significant association with likelihood of infectivity to mosquitoes both in patient's own serum and naïve serum. This is different from our previous study conducted at Adama with a very strong association observed in the likelihood of infectivity between gametocyte densities but no parasite density (Chali *et al.*, 2020). One of the reason behind this difference may be that population living in different endemic settings have different degree of exposure to malaria parasite and develop antibody against sexual stages of parasite that interfere parasite's transmission dynamics.

Importantly, for the evaluation of transmission reduction, a reduction in the oocysts number as measured by a so called R value, which is obtained by comparing the oocysts level resulting from the use of a test serum with that obtained with a control serum, was used to classify the sera as

blocking sera, reducing sera or non-blocking sera (Lensen *et al.*, 1996). From our current experiments majority of the sera were found to be reducing sera and few were non reducing sera whereas no blocking sera found comparable to previous studies as plasma from adults living in an area of malaria endemicity confirmed that naturally acquired antibodies targeting recombinant Pfs230 region had transmission reducing activities (Acquah *et al.*, 2019).

Mendis *et al*.1987 showed that Sri Lankan individuals with acute *P. vivax* infections produced gamete specific antibodies, and that antibodies from these patients inhibited transmission in direct membrane feeding assays as observed in our recent studies. On the other hand, in spite of few investigations and poor understanding, low titers of antibodies were shown to enhance human-to-mosquitoes transmission (Stone *et al.*, 2019). Although not naturally induced, antibodies against post-fertilization parasite stages such as P25 (Miura *et al.*, 2007) and P28 (Hisaeda *et al.*, 2000) also showed potential reduction in human-to-mosquito transmission.

Therefore, these antigens can serve as potential candidates for transmission blocking/reducing vaccines. However, it may take long for these vaccines to be ready for use. This is, primarily, due to the gap in our current understanding related to the prevalence, robustness, kinetics and blocking/reducing/enhancing potential of the antibodies against these antigens in different endemic settings requiring further studies.

One of the limitations to our current study was the small sample size to cover more data that may include blocking sera even if it is enough to present relevant information on transmission reducing activity of some sera.

# 7. CONCLUSION

The results of the present study indicate that whole blood own serum fed mosquitoes developed lower oocysts density whereas naïve serum replaced fed mosquitoes showed higher density of oocysts and high percentage of infection. This is an indication that there are antibodies from naturally infected individuals in malaria exposed populations in Ethiopia that act as a transmission reducing immunity in our study. The parasite and gametocyte density was not correlated to percent of infectivity that may need to explore more with increasing sample size.

# 8. RECOMMENDATION

Further study on transmission reducing antibodies for their prevalence, robustness, kinetics and blocking/reducing/enhancing potential against selected antigens in different endemic settings required. The National malaria control program should incorporate the transmission blocking strategies for successful elimination in selected areas and promote more studies for its effectiveness.

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# 10. ANNEXES

**Annex-I: Parental/guardian information and consent form for children between 5-11 years**

**Parental/guardian information**

**Title of study:** Comparison of the effect of patient's own serum versus naïve serum on *P. vivax* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia**.**

**1) Purpose of the study**

Ethiopia has enjoyed a remarkable decrease in malaria incidence and mortality in the last two decades. However, it is not clear if this success will continue and if malaria may disappear from Ethiopia or if malaria may persist. One challenge for malaria elimination is that mosquitoes can become infected after biting a malaria-infected individual. The transmission intensity is different among individuals having the clinical sign and symptom of malaria due to the immunity developed as a result of exposure to mosquitoes carrying plasmodium parasite. Exposure to sexual stage parasite will result in the production of immunity that will prevent the next developmental stage of parasite in the mosquito mid gut and prevention of malaria transmission. One of the major gaps is about how these sexual stage immunities can affect the transmission of plasmodium parasite from human to mosquito that will help in the development of transmission blocking vaccines. It is currently unclear to what extent these sexual stage immunity determines the transmission of parasites from human-to-mosquitoes. In this study, we aim to measure comparatively the effect of sexual stage immunity on plasmodium parasites transmission from naturally infected gametocyte carriers to *A. arabiensis* using serum replacement membrane feeding assay.

This study provides highly relevant information for national malaria control and elimination efforts in Ethiopia and elsewhere. Every study comes with a discomfort for participants. We have done our best to minimize this discomfort and all procedures will adhere to international guidelines of Good Clinical Practice.

We ask your permission to allow us venous blood samples from your child. These samples will only be used for research related to effect of immunity on malaria transmission. In case of further research on stored samples, all identifying information such as your name or address will be removed from the data and ethics approval will be sought.

**2) Study procedures**

This information sheet will be delivered by one of the members of the study team. Completion of this form requires a maximum of 20 minutes.

Your child will be asked to donate venous blood sample of 5mL (1 tea spoons) once. This procedure will be carried out from January to June 2020. Informed written consent will be obtained from study participants by one of the members of the study team. Blood samples will be collected by trained Medical Laboratory technologist/technician or nurses.

**3) Voluntary participation**

Your decision not to participate will not affect the care your child will receive in any way. If you chose for your child not to participate, he/she will have access to the same level of clinical care from the study.

**4) Discomforts and Risks**

Your child might feel a small amount of discomfort during blood sampling and your child may have a small amount of bruising or bleeding where the blood sample was taken. We will use sterile equipment to collect the blood sample and the small wound that may arise from the procedure will be treated adequately. The volume of blood is too small to influence your child's health and the blood will quickly be replaced by your child's body.

**5) Benefits**

If your child requires special attention, it will be linked to the nearby health service offering institution. You or your child will not be paid for participation in this study but will receive some household goods like sugar, rice and flour as compensation for the time you or your child are spending on this study and also a long-lasting insecticide treated bednet will be given free of charge. The compensation package will be one of the goods mentioned and bednet that will on average cost 100 ETB.

**6) Confidentiality statement**

The records concerning your child's participation are to be used only for the purpose of this research project. Your child's name will not be used on labels on laboratory specimens or in any report resulting from this study. At the beginning of the study, we will give your child a study identification number and this number will be used on the forms and on the laboratory specimens. Any information obtained in connection with this study will be kept strictly confidential and under lock and key. Only senior members of the study team will have access to information linking your child's name with your child's study number. During the process of publication, we will share anonymous data on study participants online. Before we share these data, all information that is confidential or may allow people to identify your child will be removed.

**7) Long term storage of samples**

We will ask your consent for long term storage of your child's samples. New techniques may become available to study the research questions we want to answer in this study. We will make your child's samples anonymous by removing the name and any identifying information. Samples may be stored at any of the collaborating institutions in Ethiopia, United Kingdom, or The Netherlands. We will only test for naturally acquired immune responses that may determine malaria transmission potential that could help to get

information for the possible elimination of malaria. If further studies are conducted on stored study material, ethics approval will be sought. In line with international regulations, we will destroy all study material after 10 years.

**8) Freedom to ask questions**

If you have any question concerning this study, do not hesitate to contact the principal investigator of the study, WAkweya Chali Gerba, Adama Science and Technology University, Adama Ethiopia and Armauer Hansen Research Institute (AHRI), Jimma Road, P.O.Box-1005, Tel. +251 (0)910120126.

**Annex-II: Parental/guardian Informed consent form for children between 5-11 years**

**Title of study:** Effect of sexual stage immunity on *Plasmodium falciparum* and *Plasmodium vivax* transmission to *Anopheles arabiensis* from naturally infected gametocyte carriers in Ethiopia.

I, _____ (Parent's/guardian's Name), having full capacity to, do hereby consent to participation of my child _____(Child's name) in the study entitled "**Comparison of the effect of patient's own serum versus naïve serum on *P. vivax* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia"** under the principal investigator Wakweya Chali Gerba. The implications of my voluntary participation, the nature, and purpose; methods and means by which it is to be conducted; and the inconveniences and hazards which may reasonably be expected have been explained to me by _____(name of research team member), and are set forth in the Informed Consent Explanation. I have been given an opportunity to ask questions concerning this investigational study, and such questions have been answered to my full and complete satisfaction. If there are further questions that may arise, I may contact Wakweya Chali Gerba (at +251910120126). I understand that I may, revoke my consent and withdraw my child from the study without prejudice at the time of the study.

I **acknowledge**/**do not acknowledge** the receipt of explanation on this Informed Consent Form (circle)

I **understand**/**do not understand** the practical consequences of this study, asking my child to give venous blood sample once (circle)

I **approve**/**disapprove** part of the sample to be analyzed outside Ethiopia (circle)

I **approve** / **disapprove** that data are shared after removing all identifying information (circle)

I **approve**/**disapprove** part of the sample to be stored for future analyses (circle). If studies are conducted using stored study material, approval from ethics committees will be sought.

I **agree**/**disagree** to be interviewed for the questionnaire (circle)

I **agree**/**disagree** to let my child participate in this study (circle)

Thumbprint if subject is unable to sign

Participant's name: _____

Parent's/guardian's name: _____

Parent's/guardian's signature: _____Date: _____

Impartial witness's name:_____Impartial witness's signature: _____Date_____

Investigator's name: _____

Investigator's signature: _____ Date: _____

**Annex-III: Information and assent form for children between 12 and 17 years**

**Participant information**

**Title of study:** Comparison of the effect of patient's own serum versus naïve serum on *P. vivax* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia

**1) Purpose of the study**

Ethiopia has enjoyed a remarkable decrease in malaria incidence and mortality in the last two decades. However, it is not clear if this success will continue and if malaria may disappear from Ethiopia or if malaria may persist. One challenge for malaria elimination is that mosquitoes can become infected after biting a malaria-infected individual. The transmission intensity is different among individuals having the clinical sign and symptom of malaria due to the immunity developed as a result of exposure to mosquitoes carrying plasmodium parasite. Exposure to sexual stage parasite will result in the production of immunity that will prevent the next developmental stage of parasite in the mosquito mid gut and prevention of malaria transmission. One of the major gaps is about how these sexual stage immunities can affect the transmission of plasmodium parasite from human to mosquito that will help in the development of transmission blocking vaccines. It is currently unclear to what extent these sexual stage immunity determines the transmission of parasites from human-to-mosquitoes. In this study, we aim to measure comparatively the effect of sexual stage immunity on plasmodium parasites transmission from naturally infected gametocyte carriers to *A. arabiensis* using serum replacement membrane feeding assay.

This study provides highly relevant information for national malaria control and elimination efforts in Ethiopia and elsewhere. Every study comes with a discomfort for participants. We have done our best to minimize this discomfort and all procedures will adhere to international guidelines of Good Clinical Practice.

We ask your permission to allow us venous blood samples from you or your child. These samples will only be used for research related to effect of immunity on malaria transmission. In case of further research on stored samples, all identifying information such as your name or address will be removed from the data and ethics approval will be sought.

**2) Study procedures**

This information sheet will be delivered by one of the members of the study team. Completion of this form requires a maximum of 20 minutes.

Your child will be asked to donate venous blood sample of 5mL (1 tea spoons) once. This procedure will be carried out from January to June 2020. Informed written consent will be obtained from study participants

by one of the members of the study team. Blood samples will be collected by trained Medical Laboratory technologist/technician or nurses.

**3) Voluntary participation**

Your decision not to participate will not affect the care your child will receive in any way. If you chose for your child not to participate, he/she will have access to the same level of clinical care from the study.

**4) Discomforts and Risks**

Your child might feel a small amount of discomfort during blood sampling and your child may have a small amount of bruising or bleeding where the blood sample was taken. We will use sterile equipment to collect the blood sample and the small wound that may arise from the procedure will be treated adequately. The volume of blood is too small to influence your child's health and the blood will quickly be replaced by your child's body.

**5) Benefits**

If your child requires special attention, it will be linked to the nearby health service offering institution. You or your child will not be paid for participation in this study but will receive some household goods like sugar, rice and flour as compensation for the time you or your child are spending on this study and also a long-lasting insecticide treated bednet will be given free of charge. The compensation package will be one of the goods mentioned and bednet that will on average cost 100 ETB.

**6) Confidentiality statement**

The records concerning your child's participation are to be used only for the purpose of this research project. Your child's name will not be used on labels on laboratory specimens or in any report resulting from this study. At the beginning of the study, we will give your child a study identification number and this number will be used on the forms and on the laboratory specimens. Any information obtained in connection with this study will be kept strictly confidential and under lock and key. Only senior members of the study team will have access to information linking your child's name with your child's study number. During the process of publication, we will share anonymous data on study participants online. Before we share these data, all information that is confidential or may allow people to identify your child will be removed.

**7) Long term storage of samples**

We will ask your consent for long term storage of your child's samples. New techniques may become available to study the research questions we want to answer in this study. We will make your child's samples anonymous by removing the name and any identifying information. Samples may be stored at any of the collaborating institutions in Ethiopia, United Kingdom, or The Netherlands. We will only test for naturally acquired immune responses that may determine malaria transmission potential that could help to get

information for the possible elimination of malaria. If further studies are conducted on stored study material, ethics approval will be sought. In line with international regulations, we will destroy all study material after 10 years.

**8) Freedom to ask questions**

If you have any question concerning this study, do not hesitate to contact the principal investigator of the study, WAkweya Chali Gerba, Adama Science and Technology University, Adama Ethiopia and Armauer Hansen Research Institute (AHRI), Jimma Road, P.O.Box-1005, Tel. +251 (0)910120126.

**Annex-IV: Parental/guardian Informed consent form for children between 12-17 years**

**Title of study:** Comparison of the effect of patient's own serum versus naïve serum on *P. vivax* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia.

I, _____ (Parent's/guardian's Name), having full capacity to, do hereby consent to participation of my child _____(Child's name) in the study entitled "**Comparison of the effect of patient's own serum versus naïve serum on *P. vivax* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia**" under the principal investigator Wakweya Chali Gerba. The implications of my voluntary participation, the nature, and purpose; methods and means by which it is to be conducted; and the inconveniences and hazards which may reasonably be expected have been explained to me by _____(name of research team member), and are set forth in the Informed Consent Explanation. I have been given an opportunity to ask questions concerning this investigational study, and such questions have been answered to my full and complete satisfaction. If there are further questions that may arise, I may contact Wakweya Chali Gerba (at +251910120126). I understand that I may, revoke my consent and withdraw my child from the study without prejudice at the time of the study.

I **acknowledge**/**do not acknowledge** the receipt of explanation on this Informed Consent Form (circle)

I **understand**/**do not understand** the practical consequences of this study, asking my child to give venous blood sample once (circle)

I **approve**/**disapprove** part of the sample to be analyzed outside Ethiopia (circle)

I **approve** / **disapprove** that data are shared after removing all identifying information (circle)

I **approve**/**disapprove** part of the sample to be stored for future analyses (circle). If studies are conducted using stored study material, approval from ethics committees will be sought.

I **agree**/**disagree** to be interviewed for the questionnaire (circle)

I **agree**/**disagree** to let my child participate in this study (circle)

Thumbprint if subject is unable to sign

Participant's name: _____

Parent's/guardian's name: _____

Parent's/guardian's signature: _____Date: _____

Impartial witness's name: _____Impartial witness's signature: _____Date:_____

Investigator's name: _____

Investigator's signature: _____ Date: _____

**Annex- V: Participant Informed assent form for children between 12 and 17 years**

**Title of study:** Comparison of the effect of patient's own serum versus naïve serum on *P. vivax* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia.

I, _____ (Participant's Name), having full capacity to, do hereby assent to my participation in the study entitled "**Comparison of the effect of patient's own serum versus naïve serum on *P. vivax* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia"**, under the principal investigator Wakweya Chali Gerba. The implications of my voluntary participation, the nature and purpose; methods and means by which it is to be conducted; and the inconveniences and hazards which may reasonably be expected have been explained to me by _____(name of research team member), and are set forth in the Informed assent Explanation. I and my parents/guardians have been given an opportunity to ask questions concerning this investigational study, and such questions have been answered to my full and complete satisfaction. If there are further questions that may arise, I may contact Wakweya Chali Gerba (at +251910120126). I understand that I may at any time, at the time of the study, revoke my assent and withdraw myself from the study without prejudice; however, I may be requested to undergo further examinations if, in the opinion of the physician, such examinations are necessary for my wellbeing.

I **acknowledge** / **do not acknowledge** the receipt of explanation on this Informed assent Form (circle)

I **understand** / **do not understand** the practical consequences of this study, asking me to venous blood samples once (circle)

I **approve** / **disapprove** part of the sample to be analyzed outside Ethiopia (circle)

I **approve** / **disapprove** that data are shared after removing all identifying information (circle)

I **approve** / **disapprove** part of the sample to be stored for future analyses (circle). If studies are conducted using stored study material, approval from ethics committees will be sought.

I **agree** / **disagree** to be interviewed for the questionnaire (circle)

I **agree** / **disagree** to let myself participate in this study (circle)

Thumbprint if subject is unable to sign

Participant's name: _____

Participant's signature: _____

Date: _____

Impartial witness's name: _____

Impartial witness's signature: _____ Date: _____

Investigator's name: _____Investigator's signature: _____ Date: _____

**Annex-VI: Information and consent form for Adult participants (older than 18 years)**

**Participant information**

**Title of study:** Comparison of the effect of patient's own serum versus naïve serum on *P. vivax* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia**.**

**1) Purpose of the study**

Ethiopia has enjoyed a remarkable decrease in malaria incidence and mortality in the last two decades. However, it is not clear if this success will continue and if malaria may disappear from Ethiopia or if malaria may persist. One challenge for malaria elimination is that mosquitoes can become infected after biting a malaria-infected individual. The transmission intensity is different among individuals having the clinical sign and symptom of malaria due to the immunity developed as a result of exposure to mosquitoes carrying plasmodium parasite. Exposure to sexual stage parasite will result in the production of immunity that will prevent the next developmental stage of parasite in the mosquito mid gut and prevention of malaria transmission. One of the major gaps is about how these sexual stage immunities can affect the transmission of plasmodium parasite from human to mosquito that will help in the development of transmission blocking vaccines. It is currently unclear to what extent these sexual stage immunity determines the transmission of parasites from human-to-mosquitoes. In this study, we aim to measure comparatively the effect of sexual stage immunity on plasmodium parasites transmission from naturally infected gametocyte carriers to *A. arabiensis* using serum replacement membrane feeding assay.

This study provides highly relevant information for national malaria control and elimination efforts in Ethiopia and elsewhere. Every study comes with a discomfort for participants. We have done our best to minimize this discomfort and all procedures will adhere to international guidelines of Good Clinical Practice.

We ask your permission to allow us venous blood samples from you. These samples will only be used for research related to effect of immunity on malaria transmission. In case of further research on stored samples, all identifying information such as your name or address will be removed from the data and ethics approval will be sought.

**2) Study procedures**

This information sheet will be delivered by one of the members of the study team. Completion of this form requires a maximum of 20 minutes.

Your child will be asked to donate venous blood sample of 5mL (1 tea spoons) once. This procedure will be carried out from January to June 2020. Informed written consent will be obtained from study participants

by one of the members of the study team. Blood samples will be collected by trained Medical Laboratory technologist/technician or nurses.

**3) Voluntary participation**

Your decision not to participate will not affect the care you will receive in any way. If you chose for you not to participate, you will have access to the same level of clinical care from the study.

**4) Discomforts and Risks**

You might feel a small amount of discomfort during blood sampling and you may have a small amount of bruising or bleeding where the blood sample was taken. We will use sterile equipment to collect the blood sample and the small wound that may arise from the procedure will be treated adequately. The volume of blood is too small to influence your health and the blood will quickly be replaced by your body.

**5) Benefits**

If you require special attention, you will be linked to the nearby health service offering institution. You will not be paid for participation in this study but will receive some household goods like sugar, rice and flour as compensation for the time you are spending on this study and also a long-lasting insecticide treated bed net will be given free of charge. The compensation package will be one of the goods mentioned and bed net that will on average cost 100 ETB.

**6) Confidentiality statement**

The records concerning your participation are to be used only for the purpose of this research project. Your name will not be used on labels on laboratory specimens or in any report resulting from this study. At the beginning of the study, we will give you a study identification number and this number will be used on the forms and on the laboratory specimens. Any information obtained in connection with this study will be kept strictly confidential and under lock and key. Only senior members of the study team will have access to information linking your name with your study number. During the process of publication, we will share anonymous data on study participants online. Before we share these data, all information that is confidential or may allow people to identify you will be removed.

**7) Long term storage of samples**

We will ask your consent for long term storage of your samples. New techniques may become available to study the research questions we want to answer in this study. We will make your samples anonymous by removing the name and any identifying information. Samples may be stored at any of the collaborating institutions in Ethiopia, United Kingdom, or The Netherlands. We will only test for naturally acquired immune responses that may determine malaria transmission potential that could help to get information for the possible elimination of malaria. If further studies are conducted on stored study material, ethics approval will be sought. In line with international regulations, we will destroy all study material after 10 years.

**8) Freedom to ask questions**

If you have any question concerning this study, do not hesitate to contact the principal investigator of the study, WAkweya Chali Gerba, Adama Science and Technology University, Adama Ethiopia and Armauer Hansen Research Institute (AHRI), Jimma Road, P.O.Box-1005, Tel. +251 (0)910120126.

**Annex-VII: Participant Informed consent form for Adults (older than 18 years)**

**Title of study**: Comparison of the effect of patient's own serum versus naïve serum on *P. vivax* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia.

I, _____ (Participant's Name), having full capacity to, do hereby consent to my participation in the study entitled "**Comparison of the effect of patient's own serum versus naïve serum on *P. vivax* infectivity to *A. arabiensis* from naturally infected gametocyte carriers using membrane feeding assay in Ethiopia**", under the principal investigator Wakweya Chali Gerba. The implications of my voluntary participation, the nature and purpose; methods and means by which it is to be conducted; and the inconveniences and hazards which may reasonably be expected have been explained to me by _____ (name of research team), and are set forth in the Informed Consent Explanation. I have been given an opportunity to ask questions concerning this investigational study, and such questions have been answered to my full and complete satisfaction. If there are further questions that may arise, I may contact Wakweya Chali Gerba (at +251910120126). I understand that I may at any time, at the time of the study, revoke my consent and withdraw myself from the study without prejudice; however, I may be requested to undergo further examinations if, in the opinion of the physician, such examinations are necessary for my wellbeing.

I **acknowledge** / **do not acknowledge** the receipt of explanation on this Informed Consent Form (circle)

I **understand** / **do not understand** the practical consequences of this study, asking me to venous blood sample once (circle)

I **approve** / **disapprove** part of the sample to be analyzed outside Ethiopia (circle)

I **approve** / **disapprove** that data are shared after removing all identifying information (circle)

I **approve** / **disapprove** part of the sample to be stored for future analyses (circle). If studies are conducted using stored study material, approval from ethics committees will be sought.

I **agree** / **disagree** to be interviewed for the questionnaire (circle)

I **agree** / **disagree** to let myself participate in this study (circle)

Thumbprint if subject is unable to sign

Participant's name: _____

Participant's signature: _____

Date: _____

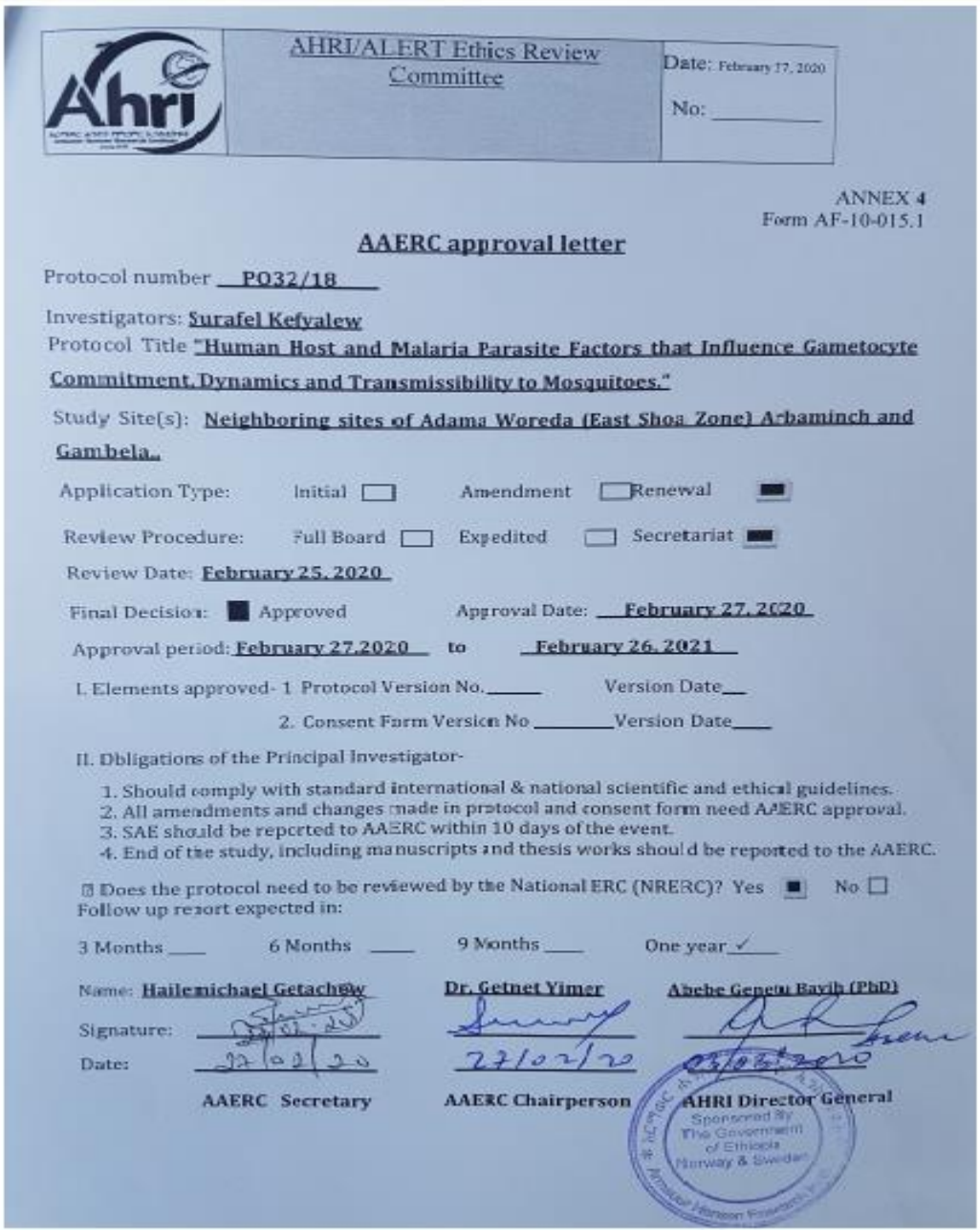
Impartial witness's name: _____

Impartial witness's signature: _____ Date: _____

Investigator's name: _____

Investigator's signature: _____ Date: _____

**Annex VIII**: AHRI/ALERT Ethics approval letter



AHRI/ALERT Ethics Review Committee

Date: February 17, 2020

No: _____

ANNEX 4
Form AF-10-015.1

## AAERC approval letter

Protocol number PO32/18

Investigators: Surafel Kefyalew

Protocol Title "Human Host and Malaria Parasite Factors that Influence Gametocyte Commitment, Dynamics and Transmissibility to Mosquitoes."

Study Site(s): Neighboring sites of Adama Woreda (East Shoa Zone) Arbaminch and Gambela.

Application Type: Initial ☐ Amendment ☐ Renewal ■

Review Procedure: Full Board ☐ Expedited ☐ Secretariat ■

Review Date: February 25, 2020

Final Decision: ■ Approved Approval Date: February 27, 2020

Approval period: February 27,2020 to February 26, 2021

I. Elements approved- 1. Protocol Version No._____ Version Date__

2. Consent Form Version No _____Version Date____

II. Obligations of the Principal Investigator-

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

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

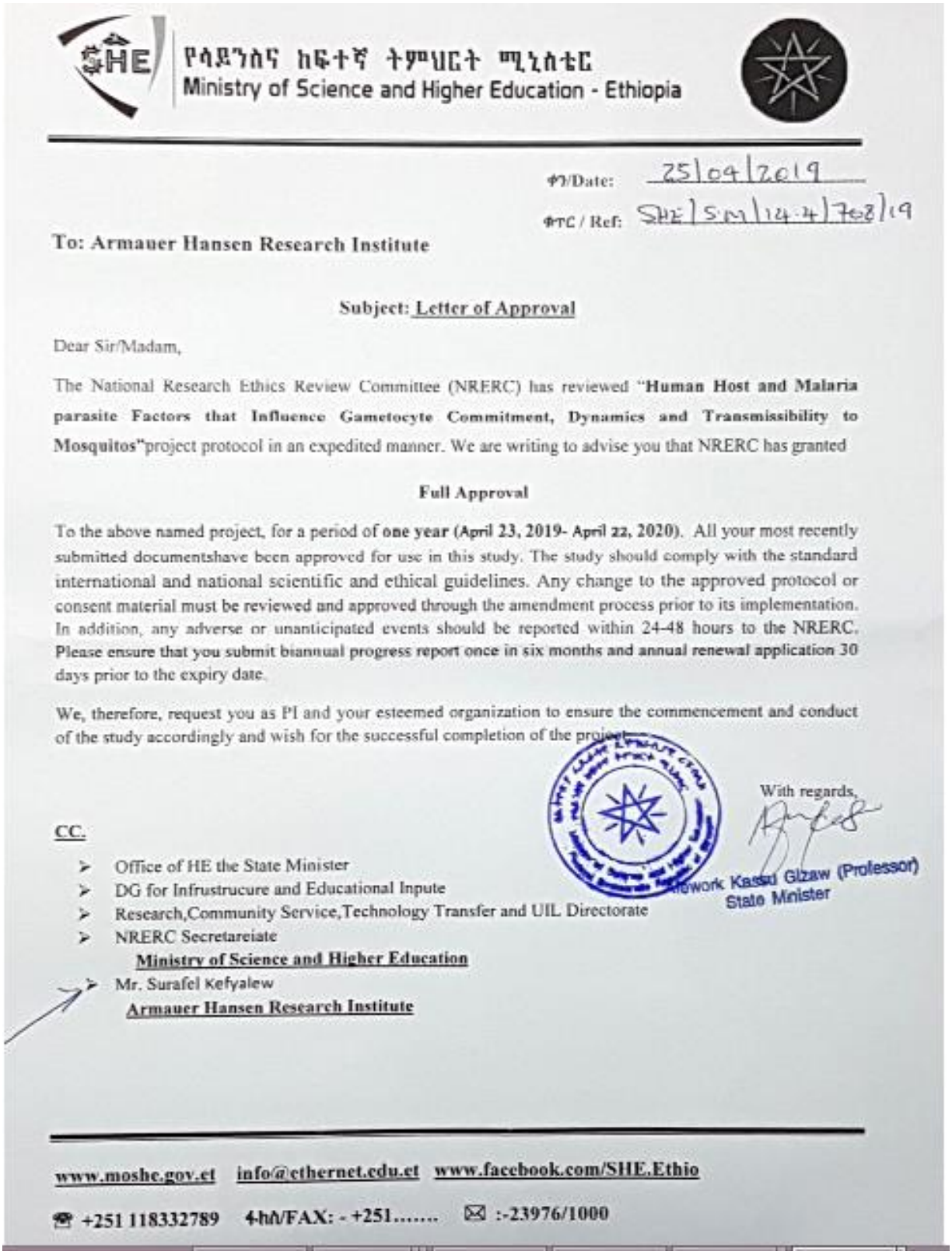
Follow up report expected in:

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

| | Hailemichael Getachew | Dr. Getnet Yimer | Abebe Genetu Bayih (PhD) |
|---|---|---|---|
| Signature: | | | |
| Date: | 22/02/20 | 22/02/20 | 03/03/2020 |
| | AAERC Secretary | AAERC Chairperson | AHRI Director General |

Sponsored By The Government of Ethiopia Norway & Sweden

**Annex IX**: National Ethics approval letter



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Ministry of Science and Higher Education - Ethiopia

ቀን/Date: 25/04/2019

ቁጥር / Ref: SHE/SM/124-4/708/19

**To: Armauer Hansen Research Institute**

**Subject: Letter of Approval**

Dear Sir/Madam,

The National Research Ethics Review Committee (NRERC) has reviewed "**Human Host and Malaria parasite Factors that Influence Gametocyte Commitment, Dynamics and Transmissibility to Mosquitos**"project protocol in an expedited manner. We are writing to advise you that NRERC has granted

**Full Approval**

To the above named project, for a period of **one year (April 23, 2019- April 22, 2020)**. All your most recently submitted documentshave been approved for use in this study. The study should comply with the standard international and national scientific and ethical guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to the NRERC. **Please ensure that you submit biannual progress report once in six months and annual renewal application 30** days prior to the expiry date.

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

With regards,

Dr. Lework Kassu Gizaw (Professor)
State Minister

CC.

- Office of HE the State Minister
- DG for Infrustrucure and Educational Inpute
- Research,Community Service,Technology Transfer and UIL Directorate
- NRERC Secretareiate

**Ministry of Science and Higher Education**

- Mr. Surafel Kefyalew

**Armauer Hansen Research Institute**

www.moshe.gov.et info@ethernet.edu.et www.facebook.com/SHE.Ethio

☎ +251 118332789 ቁጥር/FAX: - +251..... ✉ :-23976/1000

**Annex –X:** Socio-demographic characteristics of study participants, ArbaMinch, Ethiopia (2020).

| CHARACTERISTICS | %(n/N) |
|---|---|
| **Age in years, median (25th – 75th percentile)** | 20 (IQR:15 - 28) |
| **Sex** | |
| **Male** | 61.1 (11/18) |
| **Female** | 38.9 (7/18) |
| Previous malaria episodes | 77.8 (14/18) |
| **Malaria prevention applications** | |
| Ownership of bed nets | 66.7(12/18) |
| Insecticide residual spraying | 50 (9/18) |
| **transmission risk factor assessment** | |
| **Roof type** | |
| Grass | 44.4 (8/18) |
| Iron sheet | 56.6(10/18) |
| **Wall Type** | |
| Wooden plastered with mud /clay | 0) |
| Mud with cement | 66.7 (12/18) |
| Iron sheet | 0 |
| Stone or brick | 33.3 (6/18) |
| **Floor Type** | |
| Soil or Earth | 77.7 (14/18) |
| Local dung | 0 |
| Cement | 22.2 (4/18) |
| Others | 0 |
| **Water Bodies** | |
| Lake | 11.1 (2/18) |
| River | 27.8 (5/18) |
| Pond | 0 |
| Stagnant water | 0 |
| Swamp | 61.1 (11/18) |