

**SERUM-DERIVED IMMUNE FACTORS AS CORRELATES OF ARTEMISININ-
BASED COMBINATION THERAPY EFFICACY IN TREATMENT OF
UNCOMPLICATED *Plasmodium falciparum* MALARIA IN KOMBEWA,
WESTERN KENYA**

BY

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DECLARATION

Declaration by the student

I declare that this thesis has never been presented for a degree in Maseno University or any other University.

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[PG/MSC/00087/2012]

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DEDICATION

I dedicate this thesis to my husband Titus Obayi and my children Barbara, Jeelyne, Georgina, Brillian and Jephthah.

ABSTRACT

Artemisinin-based combination therapy (ACT) is recommended first-line treatment for malaria in a number of Sub-Saharan African (SSA) countries. Recent reports of decline in efficacy of ACT and emergence of ACT resistant *Plasmodium falciparum* isolates has raised global health concern. The underlying mechanisms for the development of resistance to ACT is however, not fully understood. Naturally acquired immunity to *P. falciparum* is associated with clinical protection against malaria and has been shown to influence efficacy of antimalarial drugs. To what extent pre-existing naturally acquired immunity to malaria affects efficacy of ACT remains to be established. The present study hypothesize that soluble immune factors present in sera of malaria-exposed (immune) individuals enhance the efficacy of ACT for treatment of uncomplicated *P. falciparum* malaria. Therefore, the study aims to determine the effect of serum-derived immune factors on *in vitro* growth of *P. falciparum* by serum from malaria immune and non-immune participants then correlate the effect of serum-derived immune factors on *in vitro* growth of *P. falciparum* in immune participants and ACT efficacy for uncomplicated malaria. To test the hypothesis, sera from participants (n=118) (i.e. immune sera) previously enrolled in a two-arm (i.e. artesunate-mefloquine or artemether-lumefantrine), randomized open-label trial conducted in Kisumu Country, western Kenya, were assessed for *in vitro* growth inhibitory activity (GIA) of 3D7 *P. falciparum* strain, then compared with pooled sera from malaria naïve volunteers (n=6) (i.e. non-immune sera). Each sample was divided into two portions from which one was heat inactivated, and GIA was performed at 10% and 1% serum concentration. Continuous variables were compared using Mann Whitney test and One-way analysis of variance with Tukey's post hoc. Spearman correlation coefficient test was used to correlate GIA and parasite clearance rate. Median parasite clearance rate was used as cut-off to assess treatment outcome, where fast clearers (n=80) had parasite clearance slope half-life (PCt_{1/2}) above the median parasite clearance rate while faster clearers (n=25) had PCt_{1/2} below the median parasite clearance rate. Serum from immune participants significantly inhibited *P. falciparum* growth compared to non-immune ($p < 0.0001$). Heat inactivation further diminished growth inhibitory activity of immune sera ($p = 0.009$). There was age-independent inhibitory activity ($p > 0.05$). In addition, GIA correlated with parasite clearance rate after adjusting with age (< 5years vs > 5years ($p < 0.0001$)). Further analysis showed significant positive correlation between GIA and faster parasite clearance in participants aged > 5years ($p = 0.02$). The results of this study suggest that serum-derived immune factors affect the efficacy of ACT for treatment of uncomplicated *P. falciparum* malaria. These findings will provide insight into improving on effective use of ACT drugs (dosage) in area where malaria is endemic.

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LIST OF ABBREVIATIONS AND ACRONYMS

3D7	Chloroquine sensitive clone of <i>P.falciparum</i>
ABO	Blood group A, B and O
ACTs	Artemisinin- based combination therapies
AIDS	Acquired immune deficiency syndrome
AL	Artemether Lumefantrine
ANOVA	Analysis of Variance
APD	Artesunate-Pyronaridine
ASAQ	Artesunate–amodiaquine
ASMQ	Artesunate Mefloquine
ASSP	Artesunate–sulfadoxine–pyrimethamine
BEI	Biodefense and Emerging Infections Research Resources Repository
BS	Blood smear
BSA	Bovine serum albumin
CellTos	Cell-Transversal protein for ookinetes and sporozoites
CQ	Chloroquine
CSA	Chondroitin Sulfate A
CSP	Circumsporozoite protein
DBL	Duffy binding like antigen
DCs	Dendritic cells
DHA	Dihydroartemisinin
DHA/PPQ	Dihydroartemisinin–piperaquine
EBA175	Erythrocytic binding antigen- 175
EBAs	Erythrocyte binding antigens
GEIS	Global emerging infectious surveillance and response system
GI	Gastrointestinal
GIA	Growth inhibitory activity
GLURP	Glutamate rich protein
GPARC	Global plan for artemisinin resistance containment
IC₅₀	50% inhibition concentration
IFN	Interferon
IL	Interleukin
ITNs	Insecticide-treated nets
KEMRI	Kenya Medical Research Institute
LSA	Liver stage antigen
LU	Lumefantrine
MPS	Malaria parasite slide
MSP1	Merozoite surface protein 1
NK T cells	Natural killer T-cells
P.f	<i>Plasmodium falciparum</i>
PBS	Phosphate-buffered saline
PC_{t1/2}	Parasite clearance slope half-life
PfAMA-1 3D7	<i>Plasmodium falciparum</i> apical membrane antigen-1 3D7

PfAMA-1-HB3	<i>Plasmodium falciparum</i> apical membrane antigen-1 HB3
PfCRT	<i>Plasmodium falciparum</i> Chloroquine resistance transporter gene
PfMDR1	<i>Plasmodium falciparum</i> multidrug resistance 1 gene
PfRh proteins	<i>P. falciparum</i> reticulocyte-binding homologues protein
RBCs	Red blood cells
RFUs	Relative fluorescence units
SOP	Standard operating procedure
SSC	Scientific Steering Committee
SSP	Sporozoite surface protein
TCM	Tissue culture media
TMB	Tetramethyl benzemidine
TNF	Tumour Necrosis Factor
US Dept	United State Department of Defense
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research
WRP	Walter Reed Projects

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CHAPTER ONE

INTRODUCTION

1.1. Background

The economic, health and social impact of malaria is profound with sub-Saharan African (SSA) countries accounting for the greatest global burden (WHO, 2016). According to the latest world malaria report released in 2017, there were 216 million cases of malaria resulting in 445,000 deaths worldwide (WHO, 2018). Majority of malaria cases (90%) and associated mortality (91%) occurred in Sub-Saharan Africa (SSA) (WHO, 2018). Furthermore, more than two thirds (70%) of all malaria deaths occur in children under 5 years of age in SSA, malaria remains a major killer disease especially in children, taking the life of a child every 2 minutes (WHO, 2018).

There are five species of the parasite belonging to the genus *Plasmodium* which causes human Malaria. *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* are the four human malaria species, which are spread from one individual to another by female mosquitoes of the genus *Anopheles* (WHO, 2014). Over the years, human cases of malaria have also been reported as a result of *P. knowlesi*, a species that causes malaria in monkeys, mostly found in South-east Asia (SEA) (Jongwutiwes *et al.*, 2011). According to Snow *et al* (2001), most deaths are caused by *P. falciparum* and *P. vivax*; while *P. ovale* and *P. malariae* cause a generally milder form of malaria which is rarely lethal in immune individuals (these refer to people who are naturally exposed and infected with *P. falciparum* parasite) compared to non-immune individual (refer to healthy naïve, *P. falciparum* negative people with no history of malaria infection) (Snow *et al.*, 2001; WHO, 2014).

In Kenya, malaria accounts for 30% of outpatient population and 19% of admissions to health facilities. *P. falciparum* malaria is the cause of morbidity and mortality with more than 70%

of Kenyan population at risk, especially in western Kenya around Lake Victoria and the Coastal region (WHO, 2014; Medline Plus, 2018; Kapesa *et al.*, 2018) most probably due to the presence of water body which is a breeding ground for mosquitoes. The survey study done in Western Kenya reported that overall prevalence for Kombewa of asymptomatic malaria parasitaemia was 38.3% and all confirmed and registered malaria cases in patients <5years were 17% to 27% and 9.9% to 20.7% for patients >5years (Kapesa *et al.*, 2018).

Malaria treatment has witnessed both significant advancements and setbacks. Chloroquine (CQ) was the most widely used antimalarial drug but the spread of resistance in SSA led to a rise in the disease burden in the 1980s (Trape *et al.*, 1989; Zucker *et al.*, 2003). Although CQ is no longer recommended for the treatment of *P. falciparum*, it is the drug of choice to treat *P. vivax* and *P. ovale*. (Alonso *et al.*, 2005; WHO, 2010).

Sulfadoxine pyrimethamine (SP) was replaced with artemisinin-based combination therapy (ACT) due to wide-spread resistance. Resistance to CQ and SP in Africa was first reported in Kisumu(Kombewa) due to higher prevalence of mutations at codons 86 and 1246 of the *pfmdr* gene (Zhong *et al.*, 2008). However, it is still being used as part of intermittent preventive treatment of malaria in vulnerable groups such as pregnant women, infants and children (Alonso *et al.*, 2005; WHO, 2010)

Artemisinin-based combination therapy (ACT) was introduced first in Asia but is now widely adopted in SSA and South America (Eckstein-Ludwig *et al.*, 2003; Joet *et al.*, 2003). Replacing ineffective, failing treatments (CQ and SP) with ACT has reduced the morbidity and mortality associated with malaria (Barnes *et al.*, 2005; WHO, 2016). The most commonly used ACT in Sub-Saharan Africa is artemether-lumefantrine (AL) (WHO, 2013). Kenya adopted AL as the first-line treatment for uncomplicated malaria following the precipitous decline in the efficacy of SP in 2006, with efficacy at baseline estimated at 96% which has not changed over the years (Ogutu *et al.*, 2009) as evidenced by Ogutu *et al* (2014)

when adherence to treatment regimen was higher in AL compared to dihydroartemisinin–piperazine (DHAPPQ) (Ogutu *et al.*, 2014)

Besides AL, WHO pre-qualified other ACTs, namely artesunate–amodiaquine (ASAQ), artesunate-mefloquine (ASMQ), artesunate-sulfadoxine pyrimethamine (ASSP), dihydroartemisinin–piperazine (DHAPPQ) and Artesunate-Pyronaridine (APD) which are also widely used (WHO, 2010). The ASMQ combination has been the first-line treatment for falciparum malaria on the western border of Thailand since 1994 (Phyo *et al.*, 2012). Artesunate was added to a failing mefloquine regimen, and the high effectiveness of the combination (>90% day 42 cure rates) has been sustained since then, although evidence suggests that effectiveness is decreasing (Carrara *et al.*, 2009).

The resistance to artemisinins is due to mutation of the Pfk13 propeller domain endowing the parasites with an increased ability to enter a quiescent state leading to parasite recrudescence as soon as drug pressure is removed (Paloque *et al.*, 2016). The current status of artemisinin resistance especially whether it has spread beyond the Greater Mekong sub-regions is threatening malaria control and elimination efforts causing a major setback to health security (WHO, 2014). Most severe potential effects would occur in SSA where the disease burden is highest, and systems for monitoring and containment of resistance are inadequate (WHO, 2014).

Acquired immunity is a key factor in determining the host-parasite interactions and hence influence treatment outcome that may enhance the efficacy of sub-optimal drug regimen and delay emergence of drug resistance (Greenhouse *et al.*, 2009; Rogerson *et al.*, 2010). Several factors contribute to treatment failure in malaria such as poor compliance, inadequate dosing, pharmacokinetic factors and/or resistance (Travassos & Laufer, 2009). Parasite load and innate host resistance to malaria have been shown to influence treatment outcome (Ndung'u *et al.*, 2006).

Studies have established association between *P. falciparum*-specific antibody responses and malaria treatment outcomes with different drug formulations (Aubouy *et al.*, 2007; Enevold *et al.*, 2007; Keh *et al.*, 2012; Diarra *et al.*, 2012) . Several studies have also been done on antibodies giving information that can guide vaccine development (Dent *et al.*,2008; Crompton *et al.*, 2010; Rono *et al.*, 2012; Wilson *et al.*, 2013; Boyles *et al.*, 2015; Adamou *et al.*, 2016) but currently there is lack of reliable knowledge on non-antibody mediated protection against malaria.

Serum contains non-antibody immune molecules such as properdin, complement system, soluble major histocompatibility (MHC) molecules, hormones and cytokines that might mediate protection against malaria (Murphy and Weaver, 2017). Properdin is the positive regulator of complement activation that stabilizes the alternative pathway convertases and tissue inflammation as well as the engulfing of pathogens by phagocytes (Hourcade, 2006). The complement system enhances the ability of phagocytic cells to clear microbes (parasites) and infected RBCs from the body, promotes inflammation and attacks the pathogen's cell membrane (Murphy and Weaver, 2017). Alternative pathway is not dependent on antibodies (Hourcade, 2006).

Studies have shown that complement plays a key role in immunity to malaria in conjunction with naturally occurring cellular and humoral constituents of blood (Boyle *et al.*, 2015). However, the role of acquired immunity to malaria and efficacy of ACT drug is not fully investigated. Understanding possible determinants of ACT efficacy and development of resistance is crucial in understanding the effect of pre-existing acquired immunity on the efficacy of ACT. This study aims to examine potential correlation between serum-derived immune factors' activity and efficacy of ACT against uncomplicated malaria in western Kenya.

1.2. Problem statement

Artemisinin-based combination therapy (ACT) is currently used as the first-line treatment for malaria in most countries. Reported incidence of resistance to ACT in SEA countries has raised concerns on the long-term use of ACT in clinical management of malaria globally. Therefore, it is essential to understand the underlying mechanism for emergence and spread of ACT resistance to guide the rational use of ACTs in endemic countries. Reported prevalence of malaria in Kombewa is 38.3%, given that emergence of CQ and SP in Africa was first reported in Kisumu within which Kombewa is located, the emergence of ACT resistance is likely to occur in the same geographical locality yet the driving factor to emergence of this resistance is unknown. Naturally acquired immunity to malaria influences treatment outcomes of some antimalarial drugs. Immune factors could influence ACT efficacy hence emergence of drug resistance. Antibody- complement inhibition has been shown as a prominent mechanism mediating merozoite invasion. Knowledge on non-antibody mediated protection against malaria is limited especially with ACTs in treatment of uncomplicated *P. falciparum* malaria. Serum contains many immune factors, which may influence the treatment outcome of antimalarial drugs. Furthermore, effect of serum- derived immune factors on the efficacy of antimalarial drugs including ACTs has not been investigated. Therefore, this study seeks to evaluate the role of whole serum-derived immune factors in enhancement of ACT efficacy in treatment of uncomplicated *P. falciparum* malaria in Western Kenya.

1.3. Study objectives

1.3.1. General Objective

To determine the role of serum-derived immune factors on ACT efficacy in treatment of uncomplicated *P. falciparum* malaria in western Kenya

1.3.2. Specific Objectives

- i. To determine the effect of serum-derived immune factors on *in vitro* growth of *P. falciparum* by serum from malaria immune participants and non-immune participants.
- ii. To determine the correlation between effect of serum-derived immune factors (GIA) on *in vitro* growth of *P. falciparum* in immune participants and Parasite clearance rate (ACT efficacy) for uncomplicated malaria.

1.3.3. Null hypotheses

- i. There is no significant difference in the effect of serum derived immune factors on *in vitro* growth of *P. falciparum* between malaria immune participants and non-immune participants.
- ii. There is no correlation between the effect of serum derived immune factors (GIA) on *in vitro* growth of *P. falciparum* in immune participants and Parasite clearance rate (ACT efficacy) for uncomplicated malaria.

1.4. Justification of the study

Malaria has a high economic, health and social impact in Sub-Saharan Africa (SSA) countries (WHO, 2016). Malaria kills thousands of persons especially children under 5 years in SSA (WHO, 2018). Artemisinin-based combination therapy (ACT) is the first-line treatment for malaria in most of SSA countries. The recent reports demonstrating decline in efficacy of ACTs and its resistance to *Plasmodium falciparum* isolates is therefore a global health concern (Phyo *et al.*, 2012). However, the underlying mechanism(s) for the development of resistance to ACT is not well understood. Acquired immunity to *P. falciparum* is associated with clinical protection and could enhance the efficacy of ACT (Rogerson *et al.*, 2010). Therefore, there is urgent need to understand determinants of ACT efficacy in anticipation of emergence of resistance. Currently, there is no report on the effects of serum-derived immune

factors to treatment of ACTs. Understanding the effect of serum-derived immune factors to treatment of ACT is important because it will yield crucial insight into the immunological determinants of ACT efficacy and guide rational use of ACT for uncomplicated malaria especially in malaria endemic settings, preventing death and economic burden associated with malaria.

1.5. Significance of the study

The findings of this study will potentially enable the identification of determinants that may assist in enhancement of ACT efficacy. By understanding the development of resistance to ACTs, this will inform both local and international policy makers regarding ACT use for the treatment of uncomplicated *P. falciparum* malaria. This study will benefit individuals living in malaria endemic areas directly or indirectly if the findings are implemented by policy and decision makers resulting in reduction of malaria transmission and eventual eradication. The results will further enhance Molecular biologists understanding on the biomarkers of ACT efficacy for the treatment of uncomplicated *P. falciparum* malaria.

1.6. Limitations of the study

The present study focused only on serum-derived immune factors and correlation to ACT efficacy due to time and financial constraints. However, additional factors may determine treatment outcome. Further studies may be required to address the gap.

CHAPTER TWO

LITERATURE REVIEW

2.1. Global malaria transmission

Malaria is still a major cause of morbidity and mortality in tropical and subtropical regions of the world leading to 216 million clinical episodes that led to 445,000 deaths globally (WHO, 2018). Many of malaria cases (88%) occurred in SSA, followed by the SEA (10%) and then Eastern Mediterranean (2%). Equally it was approximated ,that most deaths (91%) in 2016 were in SSA, followed by SEA (7%) and then Eastern Mediterranean (2%) (WHO, 2018)).

In addition, more than two thirds (70%) of all malaria deaths occur in children under 5 years of age in SSA, taking the life of a child every 2 minutes (WHO, 2018).. In 2015, 91 countries had ongoing malaria transmission, putting 3.2 billion people at risk (Figure 1) (WHO, 2016).

Although prevention has become an ever more vital strategy for control and eradication of malaria in developing countries, effective treatment outcome remains a fundamental component of malaria eradication (Tanner *et al.*, 2015).

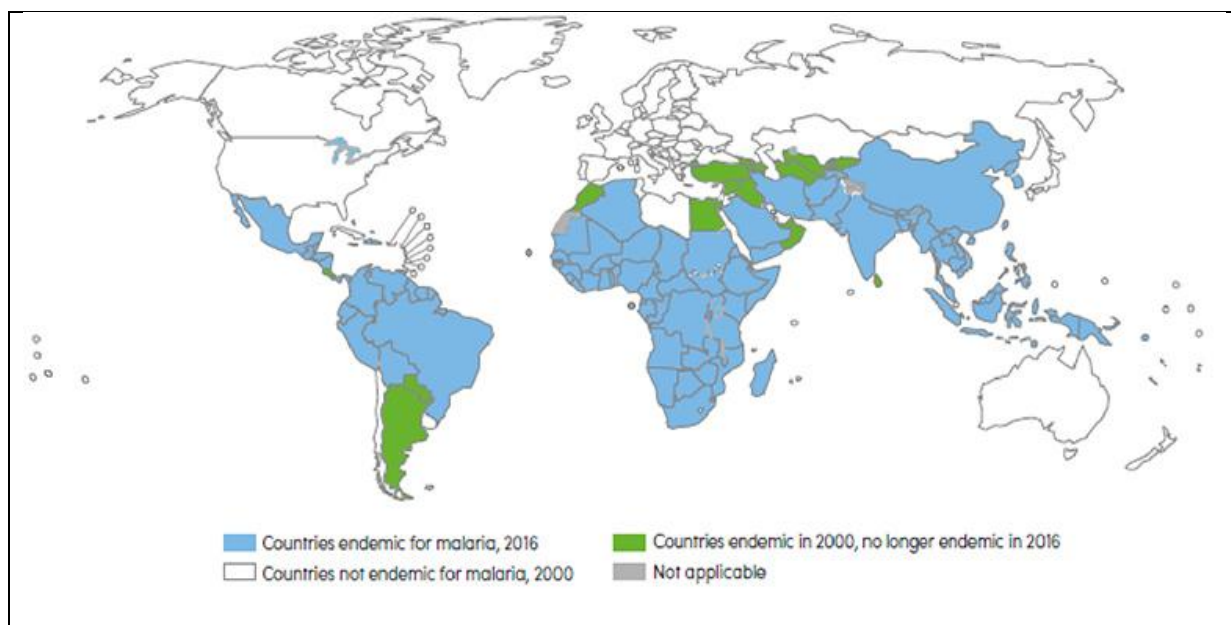


Figure 1: A map showing countries endemic for malaria between 2000 and 2016 (Adapted from WHO, 2016).

Funding for malaria control and elimination efforts has levelled off since 2010, with US\$ 2.7 billion invested in malaria programmes globally in 2016 (WHO, 2018). In SSA malaria case management cost is averagely US\$ 300 million each year (WHO, 2015). Therefore, greater awareness of threat and development of system to minimize increased number of cases are key to malaria control and reduction of increased risk (WHO, 2015). In 2015 the government endorsed a strategy specifically to deal with this threat by 2015 to 2030, since three years from 2012 to 2015 six countries reduced malaria cases by 54% and death by 84%, but there is still a long way to go to achieve eradication, however new means of treatment are urgently needed (WHO, 2016).

2.2. Etiology of malaria

Malaria is a potentially life-threatening disease caused by infection with *Plasmodium* protozoa transmitted by infective female anopheles mosquito (WHO 2014). There are about 400 different species of Anopheles mosquitoes, but only 30 of these are vectors of major importance (DaRe *et al.*, 2007; Collins *et al.*, 2012). There are five species of the parasite belonging to the genus *Plasmodium* which causes human Malaria namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (Jongwutiwes *et al.*, 2011) According to Snow *et al* (2001), most deaths are caused by *P. falciparum* and *P. vivax*; while *P. ovale* and *P. malariae* cause a generally milder form of malaria which is rarely lethal in immune individuals compared to non-immune individual (Snow *et al.*, 2001)).

2.3. Uncomplicated malaria

Malaria can be categorized as uncomplicated or complicated (severe) malaria (WHO, 2013). Uncomplicated malaria is symptomatic infection without signs of severity or evidence of vital organ dysfunction (WHO, 2013). A treatment drug for uncomplicated malaria should effectively eradicate parasites or reduce the parasite biomass to an acceptable point that the body can cope with the infection by its own immune defence, which is not the case currently

(White, 2002). Symptoms of malarial infection are nonspecific and may manifest as a flulike illness with fever, headache, malaise, fatigue, muscle aches, diarrhoea and other gastrointestinal (GI) symptoms (White, 2002). In this case, immune individuals may be completely asymptomatic or may present with mild anaemia but non-immune patients may quickly become very ill (Walther *et al.*, 2006).

Policy-makers find it hard to get an affordable and effective treatment which can rapidly control parasite resistance (Winstanley, 2000; Bioland, 2001). Chemotherapy and chemoprophylaxis are the primary means of fighting malaria parasite infections in the human host. Synthetic antimalarial drugs were introduced 70 years ago and only a small number of compounds have been found suitable for clinical use, and this limitation has been severely compromised by the spread of drug resistant parasite strains (Winstanley, 2000; Bioland, 2001).

Resistance develops by the selection of spontaneous mutations which give survival advantage in the presence of the drug, antimalarial drugs such as CQ and SP (WHO, 2005; Zhong *et al.*, 2008) with long half-life elimination phases favour this selection process (Watkins & Mosobo, 1993) where resistant parasites survive and multiply when the concentrations of drug in the blood of the patient are below the threshold required to prevent multiplication rate. Therefore it is of the essence to keep an eye on different partner drugs used like ACTs (WHO, 2005b) drugs with Short half-life with high efficacy needs to be protected from longer acting partner drugs to prevent development of artemisinin resistance (WHO, 2005b).

According to WHO (2015) children and adults with uncomplicated *P.falciparum* should be treated with ACTs, because it is a combination of a rapidly acting artemisinin and a longer acting partner drug. In these case artemisinin components rapidly clears parasite from blood and acts against the sexual stage of parasites that mediates onwards transmission to mosquitoes while the longer acting partner drug clears the remaining parasites and provides

protection against development of resistance to the artemisinin derivatives or post treatment prophylaxis. The five ACTs recommended for treatment of uncomplicated *P. falciparum* malaria are AL, ASMQ, ASSP, DHAPPQ and ASAQ (WHO, 2015).

2.4. Malaria immunity

The complex interplay of parasite proteins with the immune system of the host has made it difficult to develop an effective vaccine against the disease (Longhorne *et al.*, 2008). During its complex, multi-stage life cycle, the malaria parasite not only expresses a great variety of proteins at different stages, but these proteins also keep changing often (Longhorne *et al.*, 2008).

2.4.1. Innate immunity

Innate immunity is an immediate inhibitory response against the introduction of the parasite to the host (Murphy and Weaver, 2017). These immediate defenses include several classes of preformed soluble molecules that are present in extracellular fluid, blood, and epithelial secretions and that can either kill the pathogen or weaken its effect (Murphy and Weaver, 2017). Acute malarial infection induces immediate, non-specific immune response that tends to limit the progression of disease, although humoral and cellular mechanisms of this 'nonspecific' defense are poorly defined (Stevenson *et al.*, 2004).

Immune cells in innate immunity include Dendritic cells (DCs), monocytes, macrophages, natural killer (NK) cells, natural killer T cells and gamma-delta T cells (Stevenson *et al.*, 2004). When DCs or monocytes and macrophages come across a pathogen they release cytokines such as interleukin (IL)-12, IL-15, IL-18, TNF- α and IFN- α/β , (Artavanis-Tsakonas & Riley, 2003).

The NK cells in peripheral blood produce Interferon-gamma in response to *Plasmodium* infected erythrocytes, leading to parasitocidal macrophage activation and

development of adaptive immune responses. Further, there is production of the pro-inflammatory chemokine, Interleukin-8, that in turn plays role in the recruitment and the activation of other cells during malaria infection (Stevenson *et al.*, 2004).

2.4.2. Acquired immunity

Acquired immunity is important in enhancing success of treatment. Children born to immune mothers are protected against disease during their 6 months of life by maternal antibodies (Perlmann & Troye-Blomberg, 2002; Denise, 2009). This passive immunity is followed by 1 or 2 years of increased susceptibility before acquisition of adaptive immunity. The risk of clinical disease increases within the first 6 months of age, depending on the transmission rate (Perlmann & Troye-Blomberg, 2002). At 3 to 4 months of age, infants become susceptible to severe disease and death (Carter & Mendis, 2002; Perlmann & Troye-Blomberg, 2002; Kumar *et al.*, 2007; Denise, 2009) .

Acquired immunity against malaria develops after infection and its protective efficacy varies depending on the characteristics of the host, transmission intensity, and number of infections suffered (Carter & Mendis, 2002). It has been graded as anti-disease immunity, anti-parasite immunity, and sterilizing immunity. Non-immune individual commonly develops an acute clinical illness with very low levels of parasitemia and the infection may progress if not treated (Langhorne *et al.*, 2008).

Anti-disease immunity develops after a couple of more infections and causes suppression of clinical symptoms even in the presence of high parasite density (Doolan *et al.*, 2009). Frequent and multiple infections slowly lead to the development of anti-parasite immunity that leads to very low or undetectable parasitemia. Although sterilizing immunity is never fully achieved it results in a high degree of immune responsiveness, low levels of parasitemia, and an asymptomatic carrier status (Carter & Mendis, 2002). This kind of immunity is

mediated directly by the presence of the parasites themselves and not previous infections (Doolan *et al.*, 2009; Carter & Mendis, 2002). On the other hand, people living in unstable endemic areas tend to acquire only partial immunity (Carter & Mendis, 2002; Perlmann & Troye-Blomberg, 2002; Kumar *et al.*, 2007; Denise, 2009).

The level of antimalarial immunity influences the clinical outcome of the disease in different locations and age groups (Abdoulaye *et al* 2003). Studies conducted by Abdoulaye and the colleagues in Mali suggested that host immunity plays a critical role in the clearance of resistant *P. falciparum* infections and that the ability to clear these resistant parasites is strongly dependent on age (Abdoulaye *et al* 2003).

The potential surrogates of acquired immunity include age, malaria transmission intensity, HIV infection and pregnancy that lead to treatment failure. Merozoite surface protein 1 (MSP1), apical membrane antigen 1 (AMA1), erythrocyte binding antigens (EBAs) and *P. falciparum* reticulocyte-binding homologues (PfRh proteins) are implicated as important targets of acquired human inhibitory antibodies (Egan *et al.*, 1999; Hodder *et al.*, 2001) . Polyclonal or monoclonal antibodies can also inhibit erythrocyte invasion *in vitro* (Narum *et al.*, 2000; Hodder *et al.*, 2001).

The host's response to the *Plasmodium falciparum* red blood cells (PfRBCs), particularly in non-immune people, can contribute to the pathology of the disease, but with immune patients the response is highly effective at moderating the clinical effects of infection and reducing mortality (Langhorne *et al.*, 2008).

Complexity of immune response that underlies naturally acquired malaria immunity is a poorly understood process that likely involves several immune effector mechanisms (Langhorne *et al.*, 2008), Recently Wilson *et al.*, found that the percent of GIA in infants increased from six months to one year as a reflection for beginning of acquired immunity

(Wilson *et al.*, 2013). A longitudinal study conducted in Mali on individual aged 2 to 10 and 18 to 25 years reported that naturally acquired and vaccine-induced *P. falciparum* specific antibodies inhibits the growth of *P. falciparum* that marginally correlated with malaria incidence after adjusting for age (Crompton *et al.*, 2010). Additionally, the study suggests that antibodies which inhibit the intra-RBC growth of the parasite contribute to but are not sufficient to confer protective immunity (Crompton *et al.*, 2010).

This was also confirmed by Boyles *et al.*,(2015) in malaria-exposed children from Kenyan and Papua New Guinea reported that majority of human antibodies require complement factors to effectively inhibit merozoite invasion. They also reported that heat inactivated serum could not effectively inhibit merozoites invasion, after incubation in normal serum there was inhibition, but sera from malaria-naïve donors could not inhibit merozoites when normal or heat inactivated (Boyles *et al.*, 2015).

A cross-sectional survey study conducted in Tanzania on plasma samples from people aged between 1-84 years reported that majority of plasma samples inhibited the growth of 3D7 parasite strain (Rono *et al.*, 2012). The significant association between GIA and risk of malaria is reported only by studies that use 3D7 parasite line as in this study and two others (Crompton *et al.*, 2010; Rono *et al.*, 2012), this shows that GIA as well as its association to protection from malaria is dependent on the *P. falciparum* line.

Similarly, a study by Chulay *et al.*, conducted on three owl monkeys immunized against the Camp strain of *P. falciparum* treated with chloroquine reported that purified Immunoglobulin G (IgG) from immune serum caused a dose-dependent, time-dependent inhibition of *in vitro* parasite growth and that heat-inactivation eliminated nonspecific inhibition by normal monkey serum without diminishing immune inhibition (Chulay *et al.*, 1981).

Hastings and Watkins (2005) stated that acquired immunity is affected by transmission intensity, suggesting that transmission intensity has an indirect effect on the development of drug resistance (Hastings and Watkins, 2005). Transmission intensity influences treatment efficacy of malaria drugs, suggesting that in low transmission settings, entomological inoculation rate is also low then acquired immunity is low, treatment efficacy is high since few parasite available can be cleared from the body (Fowkes *et al.*, 2016). In this context, the rate at which immunity is acquired is low.

A longitudinal study conducted by Ataide and colleagues in Thailand reported that a population with low levels of blood stage immunity would not effectively eliminate mutant parasite which then transmit resistant parasite that influence the immunity, hence Reductions in malaria transmission decrease naturally acquired immunity (Ataide *et al.*, 2017).

Adults living in high transmission region acquire partial immunity to infection but children living in the same region and adults from low transmission settings, lack protective immunity (Djimde *et al.*, 2003). Perhaps that is why infection of non-immune individuals leads to symptomatic disease (Langhorne *et al.*, 2008), whereas immune individuals often clear infection with *P. falciparum* without drug treatment (Djimde *et al.*, 2003).

2.4.3. Cellular immunity

Cellular immune response involves the adaptive immune response in which antigen-specific T cells play the main role in controlling and eliminating the infection. It is essential to induce strong cellular immunity against target blood-stage antigens, in conjunction with functional antibodies to control pathogenic stage of the parasite's life cycle (Walther *et al.*, 2006).

CD4⁺ T-cells are essential for immune protection against asexual blood stages in human malaria as well as providing essential help for B cells to polarize induction of cytophilic IgG

subclasses that mediate antibody-dependent cellular inhibition *via* monocytes or antibody-dependent respiratory burst *via* neutrophils (Walther *et al.*, 2006).

In addition, splenic macrophages are activated for improved opsonisation of infected RBCs as well as production of pro-inflammatory parasiticidal cytokines (Walther *et al.*, 2006). CD8 T-cells have important effector functions in pre-erythrocytic immunity which contribute to protection against severe malaria as well as targeting hepatocytes harbouring developing merozoites at the late liver stage and may regulate immunosuppression in acute malaria and down-modulate inflammatory responses (Walther *et al.*, 2006).

2.4.4. Humoral immunity

Humoral immunity is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins and certain antimicrobial peptides (Janeway, 2001). Humoral immunity involves substances found in the humours, body fluids, antibody production and the accessory processes like Th2 activation and cytokines production, germinal center formation and isotypes switching, affinity maturation and memory cells generation (Janeway, 2001).

IgG1 and IgG3 are cytophilic and T cell dependent, have high affinity for Fc receptors, and mediate phagocyte activation and complement fixation (Metzger *et al.*, 2003). It has been suggested that IgG3 is more efficient at mediating these processes (Metzger *et al.*, 2003). The study conducted by Ndungu in Kilifi Kenya found out that acquisition of clinical immunity in subjects exposed to endemic malaria correlate with a qualitative change in IgG subclass antibodies to *P. falciparum* and different merozoite antigens induce relatively different levels of IgG1 and IgG3 (Ndungu *et al.*, 2002). It involves a gradual age-dependent switch from IgG 2 to 1Gg1 and IgG3 subclass (Ndungu *et al.*, 2002).

A study by Dent and colleagues in Western Kenya on the relationship between *P. falciparum* protein microarray and antibodies revealed that the overall magnitude and breadth of antibody responses among adults was greater than that in children (Dent, *et al* 2008). This confirms that antibodies develop gradually after years of exposure to the parasite (Dent, *et al* 2008)

2.5. Determinants of antimalarial efficacy and resistance

According to Galandrin and colleagues, efficacy of a drug is generally determined by the drug's ability to promote a quantifiable biological response (Galandrin *et al.*, 2007). Therapeutic efficacy studies are prospective evaluations of patients' clinical and parasitological responses to directly observed treatment for uncomplicated malaria, These studies are used as an alert to emergence of drug resistance (WHO, 2009).

The efficacy of antimalarial drugs is determined by the level of parasite susceptibility, antimalarial drug bioavailability or pharmacokinetics and host factors including immunity. Host immunity influence antimalarial drug efficacy, it has been pointed out that declining levels of immunity may have contributed to the decreased clearance rate observed in SEA and have been confused with changes in drug sensitivity level (Dondorp *et al.*, 2013; Krishna & Kremsner, 2013).

In a longitudinal study conducted by Ataide and colleagues reported that immunity to *P. falciparum* predominantly declined prior to the emergence and expansion of artemisinin resistance due to declining malaria transmission (Ataide *et al.*, 2017).

2.6. Drug pharmacokinetics and pharmacodynamics

Watkins and Mosobo (Watkins *et al.*, 1993) highlighted the importance of antimalarial pharmacokinetics, notably, the drug's terminal elimination half-life $t_{1/2}$ in determining the selection pressure driving the spread of resistance. As high-grade resistance to antimalarial drugs is usually a stepwise process and rarely occurs with a single genetic, de novo selection

is more likely to occur when a large infecting parasite population is exposed to sub-therapeutic concentrations of a single antimalarial drug (White, 2002).

The resistant parasite will survive and multiply when the concentrations of drug in the blood of the patient are below the threshold required to prevent multiplication rate of the resistant parasites. Eventually the resistant population re-expand as the drug is eliminated and concentrations fall further, causing a recrudescence of the infection and, critically, producing gametocytes for transmission (Barnes *et al.*, 2005).

2.7. Immune responses

The immune response is how your body recognizes and defends itself against bacteria, viruses and substances that appear foreign and harmful by the immune system destroying them (Medline Plus, 2018).

2.7.1. Immune response and antimalarial drugs

Advancement of antimalarial drug resistance is a major threat for malaria control. The efficacy of antimalarial drugs is determined by the level of parasite vulnerability, antimalarial drug bioavailability and pharmacokinetics and host factors including immunity. Positive contribution of host immunity to the therapeutic response to antimalarial drugs has been recognized for nearly a century (Langhorne *et al.*, 2008). Immune individuals are more likely to respond well to antimalarial treatment and require shorter treatment regimens even when drug-resistant parasites are present; reviewed in (Rogerson *et al.*, 2010).

In vivo resistance of *P. falciparum* against antimalarial drugs is determined by rapidly increasing, persistent, or recurrent parasitemia. Lack of clinical response after treatment depends on not only the ability of the parasite to escape antimalarial drugs via resistance mechanisms, but also host factors such as the immune responses that affect *P. falciparum* survival (Wellems & Plowe, 2001). Antimalarial drugs should be effective in patients from

all age groups, but studies done in Gabon (Kremsner *et al.*, 1994) showed an association between the age of the patient and the degree of therapeutic success. In Gabon increased levels of anti-MSP1 IgG1 were associated with improved efficacy of SP in children (Aubouy *et al.*, 2007).

Study conducted in Thailand by Monatrakul and colleagues (Monatrakul *et al.*, 2010) on the effects of immune plasma on parasite growth and susceptibility of *P. falciparum* to quinine and artesunate revealed inhibition of parasite development and multiplication thus increases *in vitro* antimalarial drug susceptibility of *P. falciparum* (Monatrakul *et al.*, 2010). Studies performed in Senegal (Robert *et al.*, 2000) and Thailand (Mayxay *et al.*, 2001) on antibody responses against asparagine-alanine and asparagine-proline repeats of the circumsporozoite protein and ring erythrocyte surface antigen, respectively, were elevated in patients who were treated successfully, compared to patients with treatment resistant infections (Mayxay *et al.*, 2001).

According to Adjuik *et al* (2002); in Lambare´ne´ (Gabon), the efficacy of amodiaquine for the treatment of uncomplicated *P. falciparum* malaria was evaluated in two randomized clinical trials. Amodiaquine was compared with artesunate-amodiaquine in one trial (Adjuik *et al.*, 2002) and with Atovaquone-proguanil in another trial (Adjuik *et al.*, 2002). Treatment success with amodiaquine was clearly associated with an increase of age among Gabonese children (Adjuik *et al.*, 2002). In the cohort of children treated with amodiaquine, to investigate the influence of pre-existing immunity to *P. falciparum* MSP-1 on treatment efficacy, a few parameters were considered in evaluating anti MSP-1 antibodies in both groups of patients (cured and non-cured); prevalence of detectable IgG antibodies and relative levels of the antibodies (Adjuik *et al.*, 2002). The prevalence of anti-MSP-1 antibodies was similar among patients with either parasitological or clinical cure after treatment revealing that humoral immune responses play a supportive role in the efficacy of amodiaquine

treatment (Adjuik *et al.*, 2002). The extracellular spaces are protected by the humoral immune response in which antibodies produced by B-cells cause the destruction of extracellular microorganism and prevent the spread of intracellular infections, neutralize the pathogen by binding on the pathogen and facilitating uptake of the pathogen by phagocytic cells (Janeway *et al.*, 2001).

According to Alker *et al.* (2007), *P. falciparum* Chloroquine resistance transporter gene (*PfCRT*) and *P. falciparum* multidrug resistance 1 gene (*PfMDR1*) mutations, for example, are associated with treatment failure, but these mutations are often found in patients who respond to treatment (Alker *et al.*, 2007). In 2014 mutations in the “propeller” region of the *P. falciparum* Kelch protein encoded on chromosome 13 (*kelch13*) were identified as a genetic marker of artemisinin resistance (Ashley *et al.*, 2014; Ariey *et al.*, 2014; Ghorbal *et al.*, 2014).

2.7.2. Immune response and ACT efficacy

Artemisinin resistance is characterised by slow parasite clearance (Noedl *et al.*, 2008; Dondorp *et al.*, 2009; Stepniewska *et al.*, 2011). The parasite clearance half-life can be estimated from frequent parasite density counts in patients with initial parasite densities of 10,000 parasites per μL of blood or greater (Flegg *et al.*, 2011). ACT resistance phenotype has been attributed to mutations in *P. falciparum* gene (PF3D7-1343700) encoding K-13 propeller domain. This is currently the best molecular marker of artemisinin resistance (Ariey *et al.*, 2014). However, studies that established this marker were all conducted in SEA (Ariey *et al.*, 2014) and studies done in SSA have documented low frequency non-synonymous mutations in the K13 propeller domain (Maiga-Ascofare & May, 2016; Kamau *et al.*, 2015).

The main possible way to increase drug effectiveness is by regimen changes, typically increasing the total dosage given to patients and/or changing dosage patterns. A study

conducted to African children on efficacy and safety reported that a six dose co-artemether regimen was more effective compared to four dose regimen (Lefevre *et al.*, 2001; Van Vugt *et al.*, 1999) weighing (5-25 kg). The co-artemether six-dose regimen, treating acute uncomplicated falciparum malaria in African children, achieved rapid parasite clearance and a high cure rate and treatment was generally safe and well tolerated (Falade *et al* 2005). Given the concern on artemisinin resistance threatening the therapeutic effectiveness of ACTs, it is imperative to properly design a surveillance strategy. Overreliance on malaria parasite clearance rates as a measure of drug effectiveness and as Surveillance tools for detecting the presence of potential artemisinin resistance should be changed (Hastings *et al.*, 2015).

According to Boyles *et al* (2015) complement plays a key role in immunity to malaria combined with naturally occurring cellular and humoral constituents of blood. Antibodies from malaria exposed individuals enhanced complement fixation on merozoites hence greater inhibitory activity and majority of human antibodies require complement factors to effectively inhibit merozoite invasion(Boyle *et al.*, 2015). Studies conducted in malaria endemic areas have reported that *P. falciparum* exposure is associated with growth-inhibitory activity (Bolad, 2003; McCallum *et al.*, 2008; Murhandarwati *et al.*, 2009; Perraut *et al.*, 2005), MSP1- specific (Murhandarwati *et al.*, 2009; Egan *et al.*, 1999) and AMA1-specific (Miura *et al.*, 2008; Nair *et al.*, 2002; Hodder *et al.*, 2001) and IgG fractions.

2.7.3. Non-antibody immune factors

Immune factors are small molecules polypeptides, which mainly secrete biological activity making regulative role in immune recognition and response to malaria infection (Hongwei, 2009). Serum proteins besides other functions regulate cellular activity, act as enzymes, complement components and protease inhibitors or kinin precursors (Sanui *et al.*, 2017). Some immune factors are not dependent on antibodies such as Soluble MHC molecules,

properdin, cytokines, complement system but they enhance the function of the antibodies when activated (Hourcade, 2006).

Soluble MHC molecules in serum regulates the immune response by competing their surface counterparts and consequently altering signal transduction pathways evoked by T-cells upon binding to the equivalent T-cell receptor (TCR) (Bakela & Athanassakis, 2017).

The complement system is a part of the immune system that enhances the ability of phagocytic cells to clear microbes (parasites) and damaged cells (infected RBCs) from an organism, promotes inflammation and attacks the pathogen's cell membrane (Murphy and Weaver, 2017). When the system is stimulated by one of several triggers, proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages (Janeway *et al.*, 2001). Cytokines are involved in regulation of inflammatory and immune responses during an infection (Dunna *et al.*, 2005).

Properdin is a positive regulator of complement activation that stabilizes the alternative pathway convertases, tissue inflammation and engulfs pathogens by phagocytes and inhibits the Factor H – mediated cleavage of C3b by Factor I (Hourcade, 2006).

The branch of complement system known as alternative pathway is not dependent on antibodies, it is activated by IgA immune complexes and bacterial endotoxins, polysaccharides and cell walls resulting in opsonins, chemotactic factors and membrane attack complex which fight malaria parasite (Hourcade, 2006).

2.7.4. Immune complexes

An immune complex, sometimes called an antigen-antibody complex, is a molecule formed from the integral binding of an antibody to a soluble antigen (Cush *et al.*, 2005). The bound antigen and antibody act as a unitary object. After an antigen-antibody reaction, the immune

complexes can activate complement deposition, opsonization (Goldsby, 2002) phagocytosis or processing by proteases.

Immune complex formation and deposition play a part in several diseases. The relevant antigens include exogenous antigens (e.g., drugs, foreign proteins, vaccines); infectious agents (e.g., bacteria such as staphylococci, streptococci, mycoplasma, treponemes; parasites such as plasmodia, Toxoplasma, Schistosoma; viruses such as hepatitis B, Epstein-Barr, cytomegalovirus); endogenous or self-antigens (Systemic lupus erythrocytic (SLE), cryoglobulinemia, tumor antigens) (Eggleton *et al.*, 2015). IgG and IgM are the two major classes of antibodies involved in immune complex formation. Thus, immune complexes composed of more than one antigen molecule and cross-linked lattice-wise by several bivalent IgG molecules are often capable of effective complement pathway activation. Similarly, immune complexes composed of IgM antibodies generally induce very effective, complement component activation (Williams, 1981). These IgG-immune complexes have been shown to be immunomodulatory and can regulate both innate and acquired immune responses. Ligation of activating Fc γ R on macrophages by IgG-immune complexes results in marked suppression of IL-12p40 production, a cytokine that plays a crucial role in Th1 differentiation (Sutterwala *et al.*, 1997).

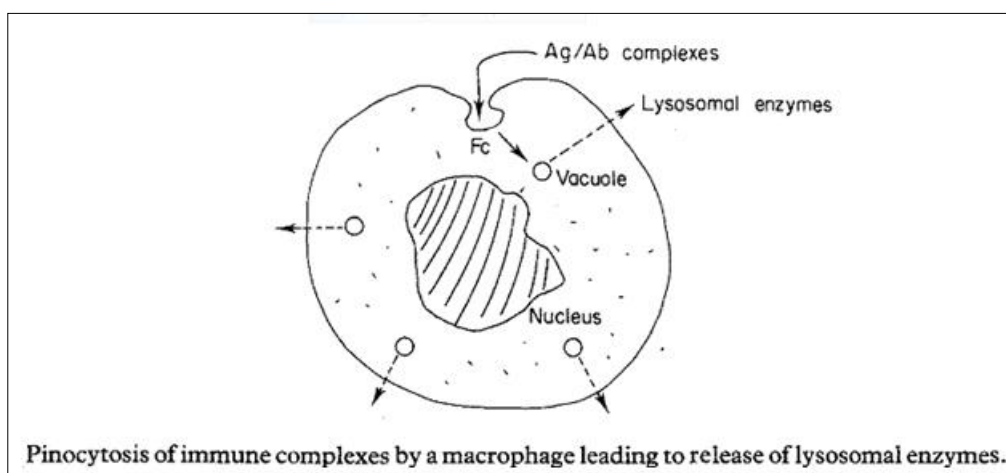


Figure 2: Role of immune complexes in malaria (Adapted from Mohammed, 1982).

Severe anaemia is one of the most lethal complications of *P. falciparum* malaria that develop from uncomplicated malaria due to antigen-antibody complexes. Red blood cells (RBCs) from children with severe malarial anaemia are deficient in complement regulatory proteins (Mohammed, 1982). A case study was carried out to determine whether these deficiencies are acquired or inherited and the relative contribution of these complement regulatory protein deficiencies, it was reported that immune complex level and the parasite density were significantly associated to the development of severe malarial anemia (Mohammed, 1982).

Immune complexes are also capable of activating various cells such as platelets, neutrophils, macrophages, eosinophils and lymphocytes, which may interfere with cell-mediated immune response (Fig 2) (Mohammed, 1982). Immune complexes have been implicated in the immunopathology of malaria infection in both experimental animal's studies and human infection (Mohammed, 1982). The role of immune complexes has been established namely malarial anaemia, cerebral malaria, 'quartan malaria nephropathy' and tropical splenomegaly syndrome in clinical situations.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Site

This project was conducted in the context of a larger trial entitled “*In- vivo* and *in-vitro* efficacy of artemisinin combination therapy in Kisumu County, Western Kenya” (PI: Dr Ben Andagalu, KEMRI/WRP, Sponsor: GEIS, US Department of Defense. This laboratory-based study was conducted using specimens obtained from patient support health facilities in Kombewa (Kisumu West) Sub-county in Kisumu County, Western Kenya (Kombewa CDC) (Appendix 1).

Under the new devolved system of government in Kenya, the HDSS area now encompasses Seme sub-county and a portion of Kisumu West sub-county. Previously, this used to cover an entire district (Kisumu West District) made up of two administrative areas (Kombewa and Maseno). It lies between longitudes $34^{\circ} 24'00''\text{E}$ and $34^{\circ} 41'30''\text{E}$, and latitudes $0^{\circ} 11'30''\text{N}$ to $0^{\circ} 11'30''\text{S}$, at an average altitude of 1400m above sea level (Tun *et al.*, 2016). Kombewa Sub-County is a 369 square kilometre rural area located near Lake Victoria in the western part of Kenya, The HDSS currently monitors a population of 141,956 individuals drawn from 34,718 households (Sifuna *et al.*, 2014). The languages spoken in this area are Luo (predominant ethnic group), Kiswahili and English. It has a long rainy season in April through June and short rains in August through October. Kombewa has perennial transmission of malaria and the most intensive transmission occurs during long and short rain seasons (Sifuna *et al.*, 2014).

Non-immune serum was obtained from naïve, *P. falciparum* negative people with no history of malaria infection in Kericho and Nairobi. National malaria control program that catalogues malaria pattern countrywide have shown five malaria zones including low risk zone that is

malaria-free (Medline Plus, 2018). Kericho and Nairobi belong to these regions, especially for natives of these areas (**Appendices 3 and 4**).

3.2. Study Design

This study is part of a two-arm randomized open-label trial study conducted from 19th March 2013 to 18th March 2014 to evaluate the role of serum-derived immune factors to ACT efficacy in treatment of uncomplicated *P. falciparum* malaria (Appendix 2). Patients presenting with uncomplicated malaria were treated with either artesunate-mefloquine (ASMQ) or artemether-lumefantrine (AL), although serum used for the study was collected (Day 0) before administration of the drugs

3.3. Ethical Approval

This study was approved by the Kenya Medical Research Institute (KEMRI) and the Walter Reed Army Institute of Research (WRAIR) institutional review boards (protocol numbers: KEMRI SSC 2518, WRAIR 1935) (Appendix 2). Personal identifiers such as name, national identification numbers were not used for data entries but the study number to maintain confidentiality (Appendix 2).

3.4. Sample Size determination

Sample size was calculated using the formula to test the equivalence between the test treatments (AL and ASMQ) (Chow *et al.*, 2003).

$$n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2 * [p1(1 - p1) + p2(1 - p2)]}{(p1 - p2)^2}$$

Where;

$Z_{\alpha/2}$ is the critical value of the Normal distribution at $\alpha/2$ (for a confidence level of 95%, α is 0.05 and the critical value is 1.96)

Z_{β} is the critical value of the Normal distribution at β (e.g. for a power of 80%, β is 0.2 and the critical value is 0.84)

p_1 and p_2 are the expected sample proportions of the two groups.

The Expected proportion for AL was 0.62 and ASMQ was 0.37 based on the proportion of patients who would remain parasitemic 24 hours after the start of treatment (Giggs *et al.*, 2016).

$$n = \frac{(1.96 + 0.84)^2 * [0.62(1 - 0.62) + 0.37(1 - 0.37)]}{(0.62 - 0.37)^2}$$
$$= 58.59 \cong 59$$

Minimum sample size required for each study arm was approximately 59 patients, which is in agreement with the WHO recommendation of a minimum sample size of 50 subjects for a single arm study (WHO, 2003). The sample size of 59 subjects per study arm allowed 118 subjects for the two study arms to be analyzed as separate single arm studies.

3.5. Sampling procedure

Patients were recruited using simple random sampling technique. A block randomization scheme with varying block size was used to prepare a randomization list of treatment assignments placed in sequentially numbered sealed opaque envelopes; each subsequently enrolled participant was allocated the next available envelope containing the treatment assignment (Artemether Lumefantrine (AL) or Artesunate-Mefloquine (ASMQ) arm) to avoid biasness (Appendix 2).

3.6. Study population

Patients residing within the study area, presenting with uncomplicated malaria were recruited.

3.6.1. Inclusion criteria

Adult/ child aged between 6 months to 65 years (minimum weight 11kg), patients presenting with a measured temperature of $\geq 37.5^{\circ}\text{C}$ or history of fever within 24 hours prior to presentation were included. Mono-infection with *P. falciparum* with baseline parasitemia of 2000 - 200,000 asexual parasites/ μl was also considered. In addition, adults who were able to provide informed consent in writing and subjects aged 13-17 years apart from parental consent were required to provide assent. However patients who were unable to read underwent consenting process in the presence of an impartial witness. Finally, willingness and ability of patient to comply with the study protocol for the duration of the study as well as being able to remain in the hospital for 3 days was included. The donors remain on the bed for 30 minutes after providing blood and served refreshments in accordance with blood donation campaign regulation (Refer appendix 4).

3.6.2. Exclusion criteria

Subjects presenting signs of severe malaria or severe anaemia defined as hemoglobin level below 6 g/dl were excluded. Also excluded patients had presence of mixed *Plasmodium* infection, mono-infection of non-falciparum *Plasmodium* or Inability to take oral medication. Furthermore those patients with history of allergy or contraindication to the study treatments were not allowed. Finally lactating or pregnant female were not allowed either including any condition that the study team felt would result in an unfavourable outcome later (**Refer appendix 4**).

3.7. Data collection

Study teams used site specific data collection tools to collect data as case report forms. These tools were completed by study teams only and no data was transferred from hospital records to study data collection document.

3.8. Sample collection

Serum samples used in this sub-study were previously collected from the patient's blood collected in efficacy study and stored at the KEMRI/WRP laboratory in Kisumu together with RBC pellets and non immune serum (Appendices 6 and 7). Blood samples were collected in an aseptic manner on day 0 by trained study staff through venous puncture. A volume of 2ml of serum was obtained upon separation from each subject and 0.5ml from each serum collected was utilized in the study (Appendices 6 and 7).

3.9. Preparation for Reagents

3.9.1. Red Blood Cells

Red blood cells were obtained from blood obtained under the blood collection protocol approved by KEMRI and WRAIR ethical review boards (KEMRI SSC 1330 and WRAIR 1919) (Appendix 3) donated by O positive donors residing in non-endemic regions (Nairobi and Kericho) who had not traveled to malaria-prone area within the previous 6 months. This blood was subjected to confirmatory ABO blood grouping tests using blood typing kit and malaria parasite slide (MPS) test. If negative for MPS, the blood was transferred to EDTA blood collecting tube and kept at 2-8°C. The RBCs were used in growing *P. falciparum* parasites.

3.9.2. Non-immune Serum

Serum was obtained from blood obtained under the blood collection protocol approved by KEMRI and WRAIR ethical review boards (KEMRI SSC 1330 and WRAIR 1919) (Appendix 3) donated by six adult males volunteers aged between 18 and 65 years with blood groups A, B and O (Pooled serum) residing in Nairobi or Kericho (non- endemic regions) and had not traveled to malaria-prone area within the previous 6 months or donated blood within the last 8 weeks (Ataide *et al.*, 2017). They had a hemoglobin level of ≥ 13 g/dL and above, tested

negative for malaria parasite slide test (MPS) and had consented to participate in the study after being subjected to blood grouping tests using ABO blood typing kit, MPS test and Haemoglobin level test. Serum enrichment was obtained from whole human blood collected in plain (anticoagulant free) blood bags from each donor, which was stored at 2-8 °C overnight to allow setting of the serum and cells portions. Serum was decanted into 50 mL centrifuge tubes, centrifuged at 8 rpm, transferred to clean 50 mL centrifuge to remove all cell components. This step was repeated three times and stored at -65 °C to -80 °C. A total volume of 1800-2000 mL of serum collected was divided into two equal portions of which one portion was heat inactivated at 56 °C for 30 minutes in water bath (Boyles *et al.*, 2015). The names of donors were not disclosed or used in recording or reporting results. Serum from males was used because woman who have had children generate antibodies to the child's allogeneic class II that can block antigen-driven responses (Nakasone *et al.*, 2016).

3.9.3. Culture media

P. falciparum in vitro culture medium was prepared as described by Johnson *et al.*, 2007 was used. Briefly, Roswell Park Memorial Institute (RPMI) 1640 basic media comprised of RPMI 1640 powder (10.4 grams; Invitrogen, Inc., Carlsbad, California, USA) combined with 2 g of glucose (Sigma Inc., St Louis, Missouri, USA) and 5.95 g HEPES (Sigma, USA) dissolved to homogeneity in 1 litre of de-ionized water and sterilized with a 0.2 µM filter. RPMI 1640 tissue culture media (TCM), for all parasite culture and drug dilutions, consisted of RPMI 1640 basic media with 0.5% Albumax II (Gibco, Grand Island, NY), 3.2% (vol/vol) sodium bicarbonate (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and 4 µg/mL hypoxanthine (Sigma Inc., St Louis, Missouri, USA). Complete RPMI 1640 media was stored at 2-8 °C.

Serum free media consisted of 1.0 mL of 1.45 mM sterile hypoxanthine to 43.4 mL of RPMI basic medium, 1.6 mL of sterile sodium bicarbonate (Thermo Fisher Scientific Inc., Waltham,

Massachusetts, USA), 7.5% and sterilized with 0.2 μM filter then put in 2-8°C. 10% complete media for Control was prepared by adding 5 mL of serum to 43.4 mL of RPMI basic medium, 1.0 mL of 1.45 mM sterile hypoxanthine, 1.6 mL of sterile sodium bicarbonate, 7.5% then sterilized with 0.8 μM , 0.45 μM , 0.2 μM filters and put at 2-8°C. Human erythrocytes and serum were obtained as described in section 3.9.1 and 3.9.2. The erythrocytes were washed three times in basic RPMI 1640 to remove white blood cells prior to use and the pellet stored at 2-8 °C.

3.10. Parasite Culture

Plasmodium falciparum- 3D7 strain obtained from Biodefense and Emerging Infections Research Resources Repository (BEI Resources) were maintained in continuous culture as previously described by Trager & Jensen (1976). Briefly, parasite samples from the cryo-freezer were thawed, washed and suspended in 5 mL TCM at 6% hematocrit in tissue culture flasks (Corning Glass Works, Corning, N.Y.). The flasks were flushed with a gas mixture consisting of 5% O₂, 5% CO₂, and 90% N₂ (Air Products Corp., Allentown, Pa.), sealed, and incubated at 37°C. TCM was changed daily and fresh erythrocytes added when required. Parasite growth was monitored by microscopic assessment of Giemsa-stained smears. Briefly thick and thin blood slides were prepared, fixed in 100% methanol, stained with 10% Giemsa solution prepared in the buffered water for 20 min as previously described (John *et al* 2004) using light microscopy. When parasitemia reached $\geq 3\%$, antigen were synchronized by sorbitol lysis (5 % D-sorbitol Sigma, St. Louis, MO) (Witkowski *et al.*, 2013) to have Ring-stage parasites only for assay. Briefly parasites were mixed and transferred into a 15 mL micro centrifuge tube, centrifuged at 3000 rpm for 10 minutes, supernatant removed, 5% D-sorbitol added (Pre-warmed to 37°C to the RBC pellet, incubated for 10 minutes at 37, vortex for 1 minute and centrifuged at 3000 rpm for 10 minutes. After aspirating supernatant, the process was repeated twice using TCM and $\geq 90\%$ ring forms was achieved.

3.10.1. Serum concentration

Serum concentration was prepared in bio-safety cabinet at ratio 1:10 and 100 μ L final volume (Dent, *et al.*, 2008). Briefly, in a tube, 10% immune CMS and 0.5 mL patient's serum was added to 4.5 mL serum free media and in another separate tube, 10% non-immune CMS was prepared by adding 0.5 mL of non-immune serum to 4.5 mL serum free media. Thereafter, 10-fold dilution of immune serum was prepared by adding 0.5 mL of 10% immune CMS to 4.5 mL of 10% non-immune CMS. In separate test tube, 0.5 mL of 10% non-immune CMS was added to 4.5 mL of 10% non-immune CMS (Appendix 5). Serum concentrations were prepared both for heat-inactivated and non-heat inactivated serum.

3.11. Growth Inhibition Assay

In vitro anti-plasmodial susceptibility assays was performed as previously described by (Dent *et al.*, 2008). Briefly, 10-fold difference of 10% CMS was done as negative controls, immune serum and non-immune serum as test controls in triplicates. Parasitemia of continuous culture $\geq 3\%$ was lowered to 1% at 2%, hematocrit, a ratio of 1:10 serum dilution in 100 μ L final volume was dispensed on Nalgene Nunc, 96 flat-bottom well cell cultures sterile with lid microtiter plates (Magna, Leicestershire, UK) and 10 μ L of 3D7 parasite was added in each well then incubate at 37°C in modular reservoirs, gassed with 90% nitrogen, 5% carbon dioxide and 5% oxygen. After 72 hrs the assays were terminated as previously described (Johnson *et al.*, 2007) and evaluation of parasite growth was done using SYBR[®] Green I nucleic acid gel stain (Appendix 5). SYBR Green I assay has been shown to have good performance at higher parasite densities (Bacon *et al.*, 2007).

3.11.1. Read out with SYBR Green I

SYBR[®] Green I nucleic acid gel stain (Sigma, Louis, MO,) with lysis buffer was subsequently added and processed as described (Johnson *et al.*, 2007). Briefly, one SYBR

Green I aliquot vial was thawed in the incubator for 5 minutes then 4 μ L of SYBR Green I were added to 2 mL of MSF lysis buffer, after which the assayed plates were placed at ambient room temperature in the dark for 24 hours. The assayed plates were examined for relative fluorescence units (RFUs) per well using a fluorescence plate reader (Tecan[®] GENios Basic plus, Tecan Group Ltd, Switzerland) Parasite replication was quantified and mean percentage growth inhibition for each serum sample calculated by an equation below;

$$\begin{aligned} \text{percentage growth inhibition} &= \\ &= \frac{\text{average RFUs in growth medium} - \text{average RFUs in patient's serum}}{\text{Average RFU in growth medium}} \times 100\% \end{aligned}$$

Optimization was done to validate the assay and control reference ranges were established upon which the assay performance was monitored using antimalarial drugs DHA and LU that have known IC₅₀ for the reference parasite strains. The drugs DHA and LU were used as positive control and tissue culture media (10% CMS) was used as negative control during the experiments.

3.12. Data management and analysis

For the larger ACT efficacy trial database was created and maintained by qualified USAMRU-K staff. The study was monitored through a reciprocal monitoring scheme whereby one of the clinical research coordinator from any of the participating sites performed reciprocal monitoring at different participating sites. Samples were de-identified and assigned numbers, code link was maintained at a separate location. Only authorized study personnel had access to the data.

The study data was obtained in Microsoft Office Excel and analyzed using Prism for windows version 5.03 (Graph Pad software, Inc, California, USA). Normality of the data was verified using Shapiro–Wilk test. Normally and non-normally distributed data were presented as mean

\pm SD and median (25th and 75th percentiles). Continuous variables were compared using the Mann Whitney test and One-way analysis of variance with Tukey's post-hoc test i.e. serum concentrations between groups and other variables such as age, gender and study arms.

In addition, correlation between GIA and baseline parasite density or GIA and parasite clearance rate was analyzed by Spearman correlation coefficient test. Parasite clearance half-life ($PC_{1/2}$) of the parasite infections in response to AL and ASMQ and baseline parasite density (Day 0) were obtained from efficacy study trial. Previous studies have used 25th quartile as cut off (Ariey *et al.*, 2014) in this population the median ($PC_{1/2}$) of 2.44 hours (interquartile range [IQR], (2.01–2.80) was used as cut-off (Ataide *et al.*, 2017) and the geometric mean (95% CI) of 33305 parasites/ μ L (95% CI = 23726 - 46750) of baseline parasite density was used as cut off to categorize participants with high parasite density and low parasite density (Tun *et al.*, 2016). Differences with a *p*-value < 0.05 were considered statistically significant.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Participants' characteristics

In this ACT efficacy trial there were two sets of study participants, the first set were patients' volunteers (immune serum) and the other entailed pooled serum from naive volunteers from Kericho and Nairobi (non-immune serum) (section 3.9.2). One hundred and eight (91.53%) out of 118 patients enrolled in AL and ASMQ were available for analysis. 53 (49.07%) were enrolled in AL and 55 (50.93%) in ASMQ (Table 1). The sum of patients aged < 5 years and 5-10 years were similar in number in both AL and ASMQ, while those above 10 years were 7 (13.21%) in AL and 9 (16.36%) in ASMQ. In both study arms, females were slightly more than males. Baseline parasite density was relatively higher in AL compared to ASMQ arm although the difference was not statistically significant ($p = 0.41$) (Table 1).

Table 1: Characteristics of patients enrolled in the two arms of the study

Characteristics	Study arm n (%)		
		AL (53)	ASMQ (55)
Age(Years)	< 5years	26(49.06%)	20 (36.36%)
	5 -10years	20(37.74%)	26 (47.27%)
	> 10years	7(13.21%)	9 (16.36%)
Gender	Female	30 (56.60%)	34 (61.82%)
	Male	23 (43.40%)	21 (38.18%)
Parasite density	Median (IQR,95%CI)	2433 (12885,133673)	31156 (854.3,117900)

Malaria episode was defined at a measured temperature of $\geq 37.5^{\circ}\text{C}$ with baseline parasitemia of 2000-200,000 asexual parasites/ μL .

4.2. Patterns of *in vitro* parasite growth inhibitory activities of serum

Naturally acquired immunity to malaria influences treatment outcomes of some antimalarial drugs. However, the extent to which pre-existing acquired immunity influences ACTs in treatment of uncomplicated *P. falciparum* malaria remains unresolved. Therefore, one of the specific aims of the present study was to determine the effect of serum-derived immune factors on *in vitro* growth of *P. falciparum* by serum from malaria immune participants and

non-immune participants. To test for this, *P. falciparum* growth inhibition were quantified for serum obtained from immune and non-immune subjects of different age groups, gender, and heat inactivated serum.

4.2.1. Serum from immune subjects inhibit parasite growth than non-immune subjects

The *in vitro* growth inhibition of *P. falciparum* in serum from immune and non-immune subjects was compared. The percentage growth inhibition of *P. falciparum* at 10% concentration for immune subjects was significantly higher than for the non-immune subjects. The mean growth inhibition at 10 % serum concentration for immune patients was 48% with median of 49% (33% - 67%) and non-immune had mean growth inhibition of 17 % with median of 24% (9% - 36%) (Figure 3A; Mann Whitney test, n =108, $p < 0.0001$). Similarly, the percentage growth inhibition at 1% serum concentration for immune patients was statistically higher than for non-immune subjects. The mean growth inhibition at 1% concentration for immune patients was 49% with median of 51% (36% - 68%) and non-immune had mean growth inhibition of 18 % with median of (2% - 37%) (Figure 3B; Mann Whitney test, n =108, $p < 0.0001$).

These results indicated that serum from immune subjects protected against *P. falciparum* than for the non-immune counterparts. The findings of the present study are in agreement with previous reports that utilized 3D7 *P. falciparum* strain (Rono *et al.*, 2012; Crompton *et al.*, 2010). However the study by Rono *et al* (2012) used plasma dialyzed against phosphate-buffered saline (PBS) and evaluated parasitemia by ethidium bromide at 1:10 dilution for subjects aged 1-84years unlike the present study where serum was used and the growth of parasite was evaluated by SYBR Green I with lysis buffer at 1:10 dilution for subjects aged 6months to 65years. Crompton *et al* (2010) used total IgG fractions purified from plasma samples using protein G columns at 6.3 mg/ml in the final test well then quantified parasitemia by lactate dehydrogenase for subjects aged 2-25years. A possible explanation to

the findings of the present study might be related to level of exposure to *P. falciparum* infection by immune subjects who then developed defense mechanisms (immunity) against the pathogen irrespective of the age group. This implies that naturally acquired immunity plays a key role in protection against *P. falciparum* malaria.

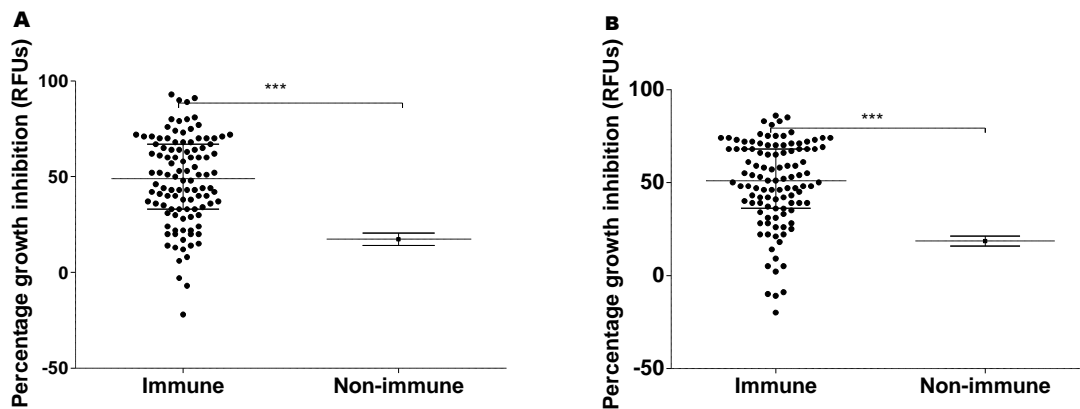


Figure 3: Comparison between *P. falciparum* growth inhibition in serum from immune volunteers and non-immune volunteers. *P. falciparum* parasite (3D7) was grown in media with serum from immune participants (n=108) and non-immune volunteers (n=6) (pooled serum from healthy naïve *P. falciparum* volunteers) prior to heat inactivation. Values represent mean growth inhibition of parasite exposed at 10% serum concentration (A) and at 1% serum concentration (B). Error bars indicate median with interquartile range. *** indicates significant difference between immune and non-immune ($p < 0.0001$) as determined by Mann Whitney test.

4.2.2. Parasite growth inhibition is independent of age of subjects

The effect of age of patients on the ability of serum to inhibit parasite growth was assessed. The patients were stratified into three age categories i.e. < 5years, 5-10 years and > 10 years (Boyles *et al.*, 2015). The percentage growth inhibition did not differ significantly among the three age categories ($p > 0.05$). Tukey's post-test further showed that there was no significant difference between the age categories at 10% serum concentration (Figure 4A; Tukey's post-test, $n = 108$, $p = 0.94$), as well as at 1% serum concentration (Figure 4B; Tukey's post-test, $n = 108$, $p = 0.79$). A trend of increasing GIA with age was observed though not significant. These findings implied that growth inhibition of *P. falciparum* was independent of age of the subjects. The current findings are consistent with several previous reports which revealed that

there is no relationship between parasite growth inhibition and age of patients (John *et al.*, 2004; Corran *et al.*, 2004; Perraut *et al.*, 2005; Wilson *et al.*, 2011; Murhandarwati *et al.*, 2009). These studies used samples from children and adults except Wilson *et al* (2011) who used children only aged 5-14years. The present study measured parasite growth inhibition by SYBR Green 1 with lysis buffer unlike most of these studies largely measured antibodies specific to merozoite surface antigens (PfMSP1-19) by ELISA. Antibodies block merozoite invasion of RBC and/or inhibit the intra-RBC growth of the parasite but are not sufficient for the acquisition of malaria immunity (Boyles *et al.*, 2015).

Given the complexity of *P. falciparum* infection and the corresponding immune response, it is likely that protective immunity requires multiple effector mechanisms. Therefore, any single assay that measures a limited subset of effector mechanisms will not provide sufficient protection from malaria (Crompton *et al.*, 2010). In this case, serum-derived immune factors contain various effector molecules giving the study an advantage over studies that measured limited effector mechanism. John *et al* (2004) looked at Gamma interferon (IFN) responses to the *Plasmodium falciparum* antigens liver-stage antigen 1 (LSA-1) and thrombospondin-related adhesive protein (TRAP).

The present findings contradict previous reports (Crompton, *et al.*, 2010; Wilson *et al.*, 2013) where GIA increase or decrease with age respectively. The contradiction could have been brought about since, Wilson *et al* (2013) measured total anti-malaria functional antibodies present in children at birth and over the following year and found that functional antibodies transferred to the fetus wane in the infants over time, Crompton *et al* (2010) as well measured purified IgG from plasma in 2-10 and 18- 25 years old individuals but the present study measured whole serum-derived immune factors in subjects aged <5years, 5-10years and >10years. A plausible explanation might be since protective effector mechanisms target specific stage of the parasite life cycle in the growth inhibitory activity assay and malaria

parasite not only expresses a great variety of proteins at different stages, but these proteins also keep changing often (Longhorne *et al.*, 2008) this might have an effect because this study used parasites at ring stage only which might not have been targeted by effector mechanisms in specific age group.

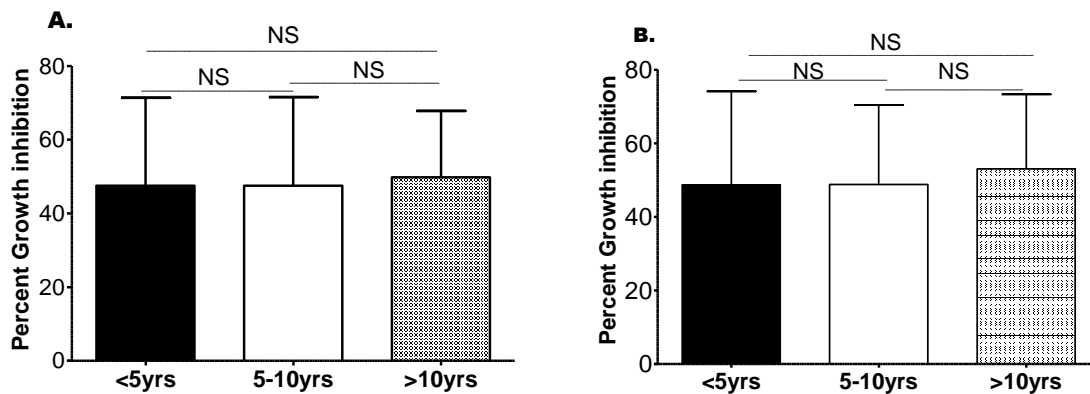


Figure 4: Comparison of parasite growth inhibition by median age groups. Values represent mean (\pm SD) of growth inhibition for participants < 5years (n = 46), 5-10years (n = 47) and > 10years (n = 15) prior to heat inactivation. Growth inhibition of parasite exposed at 10% serum concentration (A) Growth inhibition of parasites exposed at 1% serum concentration (B). Statistical comparisons between age groups were performed by One-way ANOVA with Tukey's post hoc (^{NS}, $p > 0.05$). There was a trend of GIA increasing with age although not significant.

4.2.3. Parasite growth inhibition is unaffected by gender

The present study further evaluated the effect of gender on the ability of serum to inhibit parasite growth. The median growth inhibition for females was slightly higher 51% (35% - 69%) than males 46% (25% - 66%) at 10% concentration and The percentage growth inhibition for females was slightly higher than males at 10% serum concentration albeit statistically insignificant ($p = 0.41$) (Figure 5A). The median growth inhibition for female was 52% (39% - 69%) and 51% (35% - 68%) in male at 1% concentration. Similarly, the difference in percentage growth inhibition for females and males at 1% serum concentration was not statistically significant ($p = 0.75$) (Figure 5B). The present findings indicated that gender does not confer advantage against *P. falciparum* infections. The findings concur with

available evidence by WHO (2007) stating that given equal exposure, adult men and women are equally vulnerable to malaria infection, except for pregnant women who are at greater risk of severe malaria in most endemic areas (WHO, 2007).

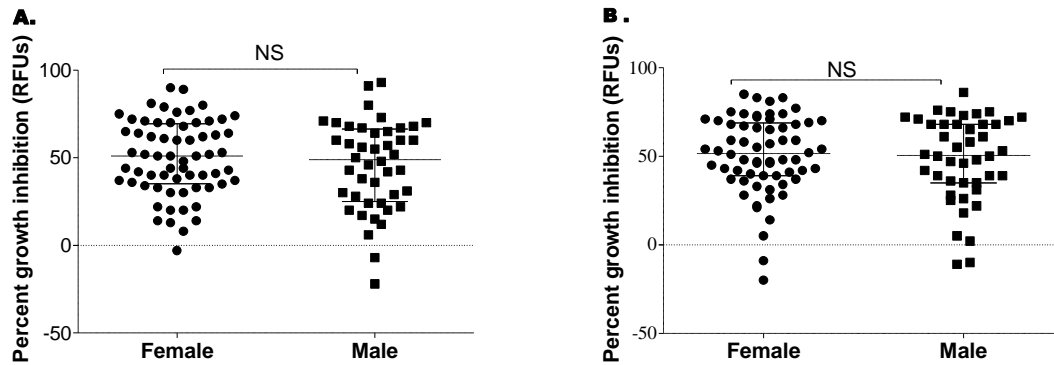


Figure 5: Comparison of parasite growth inhibition categorized by gender: Values represent mean growth inhibition for male (n=44) and female (n=64) prior to heat inactivation. Percentage growth inhibition of parasite exposed at 10 % (A) and at 1 % serum concentration (B). Error bars indicate median with interquartile range. ^{NS} indicates no significant difference between female and male ($p > 0.05$) as determined by Mann Whitney test.

4.2.4. Effect of heat inactivation of serum on *in vitro* parasite growth

The present study evaluated the effect of heat-inactivated serum from immune subjects at 10% and 1% concentration. Heat inactivation of serum at 56 °C for 30 minutes inhibited alternative complement cascade by deactivating factor B but leaves the classical complement cascade intact (Boyles, 2015). There was no statistically significant difference in percentage growth inhibition between heat inactivated serum and non-heat inactivated serum at 10% serum concentration ($p = 0.18$). Conversely, the percentage growth inhibition was significantly higher for non-heat inactivated serum than heat inactivated serum at 1% serum concentration ($p = 0.009$) (Table 2). The results suggest that alternative pathway does not account for parasite growth inhibition and classical complement cascade is more potent when the complex molecules are dissociated to 1% serum concentration. Further, the growth inhibition by pooled serum from non-immune subjects was determined between heat-inactivated and non-heat inactivated serum at 10% and 1% serum concentration. The

percentage growth inhibition for heat inactivated serum was statistically lower than non-heat inactivated serum at 10% serum concentration ($p = 0.016$). In contrast, the percentage growth inhibition between heat inactivated and non-heat inactivated serum at 1% serum concentration was not statistically different ($p = 0.32$). At this point it was noted that in non-immune pooled serum significance difference was seen between non-heat and heat inactivated serum at 10% (Table 2) suggesting that the classical complement cascade is part of the innate immune system (Janeway *et al.*, 2001) and plays role in parasites growth inhibition. The data also showed that there was no difference in percentage growth inhibition between heat-inactivated and non-heat inactivated serum drawn from different age groups ($p > 0.05$) (Table 2). Taken together, the findings of the present study pointed out that parasite growth inhibition by immune serum was not age- dependant. On the other hand the growth inhibition of parasites by immune serum at 10% was lower compared 1% suggesting a dose-dependent effect of effector molecules in all the age groups.

Dose-dependence in this case suggests that immune effector molecules are more potent at lower concentration when molecules are dissociated (diluted) from complex state in order to respond effectively to pathogens hence higher inhibition of the parasite growth (Mlambo & Kumar, 2007). The present findings concur with previous reports (Boyles *et al.*, 2015; Mlambo & Kumar, 2007; Chulay *et al.*, 1981) as they all used heat-inactivated serum at 1:10 dilution and then cultured 3D7 parasites strain. However, the previous studies by Boyles *et al* (2015) used purified immunoglobulin G (IgG) at 1:200 and 1:10 testing for merozoites invasion using ELISA for complement fixation (Boyles *et al.*,2015); Chulay *et al* (1981) used serum from three owl monkey, for Camp strain of *P. falciparum* then tested growth inhibition *in vitro* by 10% serum (Chulay *et al.*, 1981) and then Mlambo and Kumar utilized dialyzed plasma samples at 10% and 20% using LDH assay and microscopy (Mlambo & Kumar, 2007). This may suggest that some immune factors contain molecules that are heat labile in

nature and are denatured by heat possibly due to high temperature they lose small molecular weight growth factors or inactivate others that may be required for *in vitro* growth of parasites.

Table 2: Effect of heat inactivation on parasite growth inhibition

Parasite growth inhibition (%)						
Immune participants (n = 108)				Non-immune (pooled sera from six <i>P. falciparum</i> naïve participants)		
Serum concentration	Non-heat activated	Heat-inactivated	p-value	Non-heat activated	Heat-inactivated	p-value
10%	48	45.4	0.18	17.8	13.2	0.016
1%	49.5	45.7	0.009	18.6	20.7	0.32

P. falciparum 3D7 clone were grown in media with heat inactivated serum (56 °C) for 30 minutes and non-heat inactivated serum from immune and non-immune participants; the values represents the mean (\pm SD) growth inhibition. Mann Whitney test was used to compare the variables. Bold *p*- value represent statistically significant.

4.2.5. Baseline parasite density and growth inhibitory activity

The study subjects were grouped into two categories based on geometric mean (95% CI) of baseline parasite density (Tun *et al.*, 2016). A baseline parasite density above the geometric mean of 33305 parasites/ μ L (95% CI = 23726 - 46750) were categorized as participants with high parasite density (n = 50) while subjects below the threshold were classified as participants with low parasite density (n = 40) (Tun *et al.*, 2016). The median (IQR) growth inhibition for high parasite density at 10% serum concentration was 50.50% (32.50% - 65.00%) with mean of 47.16% while median (IQR) of 54% (30.75% - 71.25%) with mean of 49.82 at 1% serum concentration. The median (IQR) growth inhibition for low parasite density at 10% serum concentration was 52.00% (30.75% - 69.50%) with mean of 49.55% while median (IQR) of 49.00% (27.00% -71.25%) with mean of 48.33% at 1% serum concentration respectively. Further, there was no correlation between GIA and high parasite density ($p = 0.73$) as well as low parasite density ($p = 0.14$) (Table 3). The current findings are in agreement with previous reports (Hastings *et al.*, 2015) that the initial parasite number has no effect on the subsequent shape of dynamics in the model output. A potential

explanation for these observations could be attributed to high immunity developed due to high entomological inoculation rate in endemic region.

Table 3: Correlation between GIA and baseline parasite density

Baseline parasite density	Value of		
	n (%)	r_s	p-value
Low	40	0.23	0.14
High	50	0.05	0.73

Prior to heat inactivation of serum, the participants were categorized based on geometric mean 33305 parasites/ μ L (95% CI = 23726 - 46750) of baseline parasite density (Day 0) as cut-off. Participants with parasite density above geometric mean had high parasite density while participants below the threshold had low parasite density, r_s = Spearman correlation coefficient as determined by two-tailed Spearman correlation coefficient test.

4.3. Growth inhibition and parasite clearance slope half-life (PCT_{1/2})

The second specific aim of the present study was to determine the correlation between effect of serum-derived immune factors on *in vitro* growth of *P. falciparum* and ACT efficacy in immune patients with uncomplicated malaria. To assess this, study subjects were grouped into two categories based on median clearance slope half-life (PCT_{1/2}). The median PCT_{1/2} of 2.44 hours (2.01 - 2.80) hours was used as cut-off time (Flegg *et al.*, 2011; Ataide, *et al.*, 2017). The patients with PCT_{1/2} above the median of 2.44 (2.01 - 2.80) hours were categorized as fast clearers while subjects below the median PCT_{1/2} were classified as faster clearers.

There was no correlation between the study cohort (n=105) and parasite clearance rate ($p = 0.53$). When analyzed based on two age groups <5 years and >5 years (Ogutu *et al.*, 2014; Kapesa *et al.*, 2018) results revealed statistically significant positive correlation for participants aged > 5 years (n=59) and parasite clearance slope half-life ($p < 0.0001$). Conversely, there was no association in patients aged < 5 years (n=46) ($p = 0.95$) (Table 4). Further correlations were done between fast and faster clearer within the age category (< 5 years and > 5 years). There was a statistically significant correlation in faster clearer aged > 5 years ($p = 0.02$). However, there was no correlation for participants aged < 5 years ($p = 0.25$) (Table 4). Taken together, the present findings indicated that serum-derived immune factors are determinants in

enhancement of ACT efficacy. Further, age influences treatment outcome. The current findings are in consonance with previous reports that used the 3D7 strain (Crompton *et al.*, 2010; Rono *et al.*, 2012). However, another study did not find such an association (McCallum *et al.*, 2008). The explanation for this observation is that parasite lines differ in their relative expression of proteins, such as *P. falciparum* reticulocyte-binding homologues 1 (PfRh1) that are necessary for inhibitory activity, 3D7 express less pfRh1 therefore more inhibited than other strains (Rono *et al.*, 2012).

Table 4: Correlation between GIA and Parasite clearance rate

Parameter		n (%)	r _s	p-value
Entire population		105 (95%)	-0.06	0.53
< 5 years		46 (44%)	0.01	0.95
Treatment outcome	Faster	9 (9%)	-0.30	0.43
	Fast	37 (27%)	0.01	0.95
>5 years		59 (56%)	0.52	<0.0001
Treatment outcome	Faster	16 (15%)	0.47	0.02
	Fast	43 (41%)	0.11	0.25

Participants were categorized by age <5years and > 5years. The median PCt_{1/2} of 2.44 hours (IQR, 2.01 - 2.80) hours was used as cut-off time. The participants with PCt_{1/2} above the median PCt_{1/2} were fast clearers while those below were faster clearers. r_s = Spearman correlation as determined by two-tailed Spearman correlation coefficient test. *p*-values in bold letters represents statistical significant correlation.

Further correlation analysis was done between GIA and parasite clearance rate (PCt_{1/2}), and median (PCt_{1/2}) was used as a cut-off. Those whose (PCt_{1/2}) was above the median PCt_{1/2} were classified as fast clearers while those below the median PCt_{1/2} were classified as faster clearers. Correlation was observed between GIA of faster clearers aged > 5 years and parasite clearance rate which agrees with a multinational study done in Southeast Asia (SEA) where higher levels of immunity were associated with faster PCt_{1/2} (Ataide *et al.*, 2017). This findings agrees with report that showed that immune individuals are more likely to respond well to antimalarial treatment and require shorter treatment regimens even when drug-resistant parasites are present (Rogerson *et al.*, 2010).

This may be attributed to study area, since Western Kenya is a high malaria transmission setting, it provides for antigenic diversity of *P. falciparum* populations causing acquisition of immunity, therefore the population aged >5 years have well-developed acquired immunity. It has been shown that the antigenic and genetic repertoires of *P. falciparum* populations are wider in high-transmission areas generated by more frequent recombination events in the mosquito (Hoffmann *et al.*, 20010. A study by Patel *et al* (2017) showed extensive diversity in the asexual stage antigens (Patel *et al.*, 2017).

The present study used whole serum, which contains wide scope of effector mechanisms that plays vital role in enhancement of ACT efficacy, most of the studies have widely looked at specific immune factor such as antibodies and complement factors in merozoite invasion (Boyles *et al.*, 2015). Moreover, this study was conducted in Western Kenya a holoendemic region where drugs resistance is likely to occur, whereas many studies on ACTs have been done in other SSA countries (Kamau *et al.*, 2015; Maiga-Ascofare & May, 2016) and SEA (Tun *et al.*, 2016). Potential explanation to the results observed may be attributed to parasite line used, GIA methods, *P. falciparum* transmission dynamics or genetic backgrounds of the study population or artefacts of study design (i.e. differences in the age distribution of the study population).

In summary, the study reported here suggests that the study population contain necessary underlying effector mechanisms that could enhance ACT efficacy in treatment of uncomplicated malaria. Although suspected specific underlying effector mechanism that predominantly play crucial role in immunity are not known, this is because the study used whole serum containing many immune factors. Complexity of *P. falciparum* infection and the corresponding immune response, calls for multiple effector mechanism characterization to specifically inform on ACT efficacy. Such information could provide novel input to the rationale use of ACTs and immunological determinants to drug resistance in endemic areas.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1. Summary

In summary, the results presented indicated that serum from immune participants significantly inhibited *P. falciparum* growth compared to non-immune ($p < 0.0001$) and GIA correlated with parasite clearance rate after adjusting for age (<5 years versus >5 years) ($p < 0.0001$). Further analysis showed significant positive correlation between GIA and faster parasite clearers in participants aged >5 years ($p = 0.02$).

5.2. Conclusions

This study reports on the immune mediators that augment ACT effectiveness in malaria endemic regions. Based on the findings presented the following conclusions can be drawn:

- i. The findings confirmed immune factors in serum from study population inhibits the growth of *P. falciparum* parasites.
- ii. *In vitro* growth inhibitory activity of the immune factors in serum from the study population correlates with parasite clearance rate as an indicator of ACT efficacy.

5.3. Recommendations

5.3.1. Recommendations for present study

- i. The findings confirmed that people in endemic regions harbor immune molecules against malaria parasites. This could inform guidelines for malaria parasites control programs, surveillance and epidemiology studies tracking and investigating the emergency and development of malarial drug resistance

- ii. *In vitro* growth inhibitory activity of the immune factors in serum from study population correlates with parasite clearance rate as an indicator of ACT efficacy. This could inform dosage recommendation and guidelines on use of ACTs in malaria endemic region.

5.3.2. Recommendations for future research

- i. There is need to determine the specific molecules in the serum that could be mediating the efficacy of ACT in malaria endemic population
- ii. There is need to explore the underlying mechanisms used by the potential molecules in the serum to enhance ACT treatment outcome
- iii. Parasite clearance slope half-life has majorly been used as a metric for drug effectiveness and resistance, other metrics such as immune status and the infected RBC clearance rates should be explored.
- iv. The sample population available was skewed towards younger age group (<10years), Therefore a sample population with older age group (>10years) would be recommended to compare with the study findings.

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APPENDICES

Appendix 1: Map showing the study site

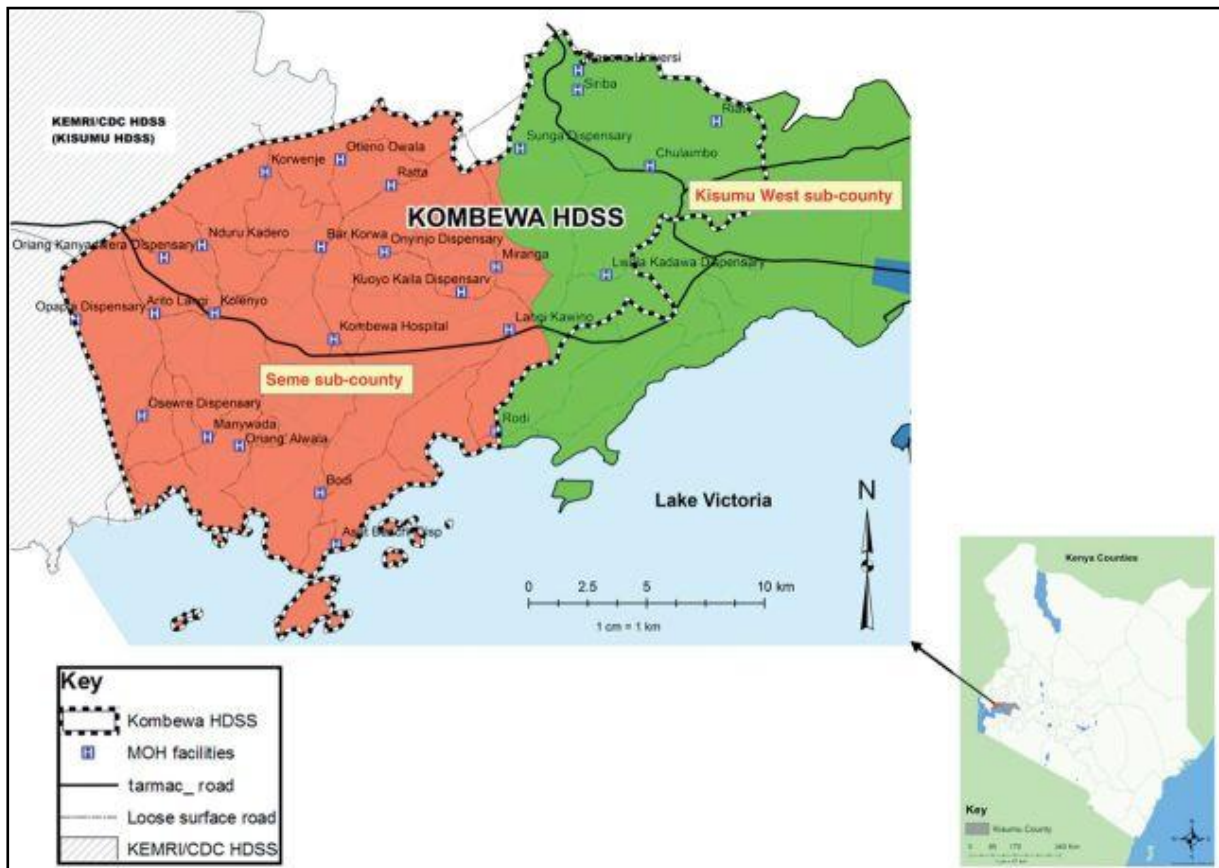


Figure 6: Map of the study area HDSS; also showing the study site (Kombewa CRC) (Sifuna *et al.*, 2014). (It lies between longitudes $34^{\circ} 24'00''\text{E}$ and $34^{\circ} 41'30''\text{E}$, and latitudes $0^{\circ} 11'30''\text{N}$ to $0^{\circ} 11'30''\text{S}$)

Appendix 2: Study Ethical clearance



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1

March 20, 2013

TO: **DR. BEN ANDAGALU,
PRINCIPAL INVESTIGATOR**

THRO': **DR. RASHID JUMA,
THE DIRECTOR, CCR,
NAIROBI**

Dear Sir,

RE: **SSC PROTOCOL NO. 2518 (INITIAL SUBMISSION): IN VIVO AND IN VITRO
EFFICACY OF ARTEMISININ COMBINATION THERAPY IN KISUMU COUNTY,
WESTERN KENYA.**

This is to inform you that during the 213th meeting of the KEMRI/ERC held on 19th March 2013, the above referenced study was reviewed.

The Committee notes that the above referenced study seeks to assess the degree of artemisinin resistance in subjects presenting with uncomplicated *P. Falciparum* malaria.

The Committee concluded that due consideration has been given to the ethical issues that may arise from the conduct of the study and granted approval for implementation effective **19th March 2013**.

Please note that authorization to conduct this study will automatically expire on **18 March 2014**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **05 February 2014**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study

Sincerely,

**DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE**

Appendix 3 Blood collection protocol approved

MCMR-UWZ-C

15 May 2018

MEMORANDUM FOR Director, Human Subjects Protection Branch (HSPB), Walter Reed Army Institute of Research (WRAIR), 503 Robert Grant Avenue, Silver Spring, Maryland 20910-7500

SUBJECT: Continuing Review Report Acceptance for the Minimal Risk Human Subjects Research Protocol, **WRAIR #1919**

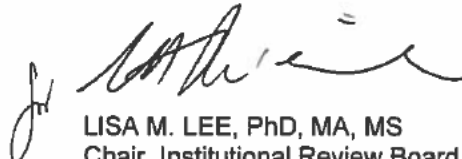
1. The continuing review report, dated 23 March 2018, for the protocol **WRAIR #1919**, entitled, "Human Blood Collection for the *In vitro* Culture of Malaria Parasites," (Protocol Version 2.7, dated 10 May 2016), submitted by Ben Andagalu, MD, MSc, Kenya Medical Research Institute (KEMRI), Kisumu, Kenya, is accepted.
2. The continuing review report covers the reporting period from 24 March 2017 through 13 March 2018. This study is open and enrolling subjects.
3. This minimal risk protocol is eligible for review by expedited review procedures per 45 CFR 46.110(b)(1) and 32 CFR 219.110(b)(1) category 2(a) as published in the 09 November 1998 Notice in the Federal Register (63 FR 60364-60367), as blood draw volumes from healthy adults do not exceed 550 ml in an 8 week period. This study also continues to meet the requirements set forth in 32 CFR 219.111 and 45 CFR 46.111.
4. The KEMRI Scientific and Ethics Review Unit (SERU) granted continuation approval of this study on 08 May 2018, with an expiration date of 11 May 2018. Please provide a copy of the updated KEMRI SERU approval letter when it becomes available to avoid an interruption in work.
5. The study continues to receive funding through the Department of Defense Global Emerging Infections Surveillance and Response System (GEIS).
6. The following documents were reviewed and are approved for continuation:
 - a. Continuing Review Report, dated 23 March 2018;
 - b. Protocol (Version 2.7, dated 10 May 2016);
 - c. Informed Consent Form (Version 2.3, dated 17 May 2012);
 - d. Recruitment Script (Version 2.0, dated 07 May 2012);
 - e. Data Collection Tool (Version 3.0, dated 25 January 2016); and
 - f. Translation documents for items c and d above (Kiswahili and Kipsigis).
7. Per the current WRAIR Policy #11-49, "Initial and Continuing Human Subjects Protection Education and Training Requirements," an 80% grade on each individual module must be obtained. The Principal Investigator (PI) is responsible for ensuring each research team member's, to include those listed on the protocol, as well as those who are not explicitly listed but may be providing study/laboratory support, human subjects protection training is current. Additionally, the PI must maintain records of documentation of this training (i.e., a staff log and training files).
8. The expiration date of this study at the WRAIR is **23 May 2019**. The PI is responsible for submitting a continuing review report to the WRAIR Institutional Review Board (IRB) and the KEMRI SERU in time for the report to be reviewed and accepted/approved prior to the respective expiration dates to avoid an interruption in work. A closeout report or a request for

MCMR-UWZ-C

SUBJECT: Continuing Review Report Acceptance for the Minimal Risk Human Subjects Research Protocol, **WRAIR #1919**

an extension must be submitted to the WRAIR HSPB no later than **23 May 2022**, as per the protocol. No changes, amendments, or addenda may be made to the protocol without prior review and approval by the WRAIR IRB and KEMRI SERU.

9. The point of contact for this action is Victoria Newman BA, at (301) 319-9588 or at Victoria.L.Newman8.civ@mail.mil



LISA M. LEE, PhD, MA, MS
Chair, Institutional Review Board
Walter Reed Army Institute of Research

CF:

Ben Andagalu, MD, MSc
Douglas Shaffer, MD
Victor Melendez, LTC, MS
Stacey Gondi
Margaret Odongo
Regulatory Affairs-Kenya

Appendix 4: Blood collection procedure

SOP Title: STANDARD OPERATING PROCEDURE FOR BLOOD COLLECTION USING PROTOCOL # WRAIR 1919	SOP No.	KSM/MDR_4615
	version	02
Effective Date:	Page	66 of 97

1.PURPOSE/ APPLICABILITY:

1.1. **Purpose:** This Standard operating procedure provides the guidelines that will be used in blood collection within the DEID program using proper techniques according to the College of American Pathologists requirements and WRAIR IRB.

1.1.1. Human blood is needed for in vitro *Plasmodium falciparum* culture and malaria drug testing. From whole blood, either serum or red blood cells are separated and used as part of media (serum) or substrate for parasite (red blood cells).

1.1.2. For this purpose, whole blood will be collected from the donors who attain the criteria IAW blood collection protocol, WRAIR # 1919.

1.1.3. It is critical that the Phlebotomist / laboratory technician is skilled and knowledgeable of the order of draw, how to take care of hematomas and the first Aid procedures in the event that the donor has an adverse medical event during or shortly after the blood has been drawn.

1.2. **Applicability:** This SOP applies to all the designated Quality Assurance Quality Control (QAQC) officers, study coordinator(s) MDR Laboratory personnel, personnel within the basic science program, laboratory manager(s), blood collection site clinicians, trainees, Principal Investigators (PIs), USAMRD-K Laboratory Officer and the DEID director

2. ABBREVIATIONS AND TERMS:

- 2.1. ACD – Acid Citrate Dextrose
- 2.2. CC – Cubic Centimetre
- 2.3. CPR – Cardiopulmonary Resuscitation
- 2.4. DEID –Department of Emerging Diseases
- 2.5. GEIS – Global Emerging Infections Surveillance and Response System
- 2.6. IAW – In Accordance With
- 2.7. IRB –Institutional Review Board
- 2.8. KEMRI – Kenya Medical Research Institute
- 2.9. Lab – Laboratory
- 2.10. MAJ. – Major
- 2.11. mL –Milliliters
- 2.12.

SOP Title: STANDARD OPERATING PROCEDURE FOR BLOOD COLLECTION USING PROTOCOL # WRAIR 1919	SOP No.	KSM/MDR_4615
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- 2.13. PI(s) – Principal Investigator(s)
- 2.14. QA – Quality Assurance
- 2.15. QC – Quality Control
- 2.16. SOP – Standard Operating Procedure
- 2.17. USAMRD-K – United States Army Medical Research Directorate Kenya
- 2.18. VCT – Voluntary counseling and Testing
- 2.19. WRAIR – Walter Reed Army Institute of Research
- 2.20. WRP – Walter Reed Project

3. MATERIALS AND REAGENTS

3.1. Materials

- 3.1.1. 60 cc Syringe(s)
- 3.1.2. 30 cc Syringe(s)
- 3.1.3. 21 gauge needles
- 3.1.4. Gloves
- 3.1.5. Gauze
- 3.1.6. Tube stand
- 3.1.7. Tourniquet
- 3.1.8. Rubber bulb
- 3.1.9. Blood collection bags without anticoagulant (The bags should be sterile)
- 3.1.10. Sphygmomanometer
- 3.1.11. Sterile cotton swabs and methylated spirit (Each of these must be placed in clean containers.)
- 3.1.12. Medicated dressing
- 3.1.13. 50 mL Centrifuge tubes

3.2. Reagents

- 3.2.1. Collection tubes with ACD

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RESPONSIBILITIES:

- 4.1. It is the responsibility of the MDR laboratory supervisor to review all SOPs.
- 4.2. It is the responsibility of all laboratory service personnel working on all protocols partially or fully being implemented by MDR laboratories to be familiar with this SOP.
- 4.3. Technical staff is responsible for the preparation, review and updating of all SOPs relative to their daily operations.
- 4.4. QAQC Officers are responsible for ensuring that all SOPs are updated annually and meet the standards outlined within this SOP.
- 4.5. Training on SOPs will be conducted upon entry into any position within DEID MDR laboratory and annually for all personnel to which the SOP applies.
- 4.6. It is the responsibility of the MDR personnel to ensure that anyone joining the MDR protocol is well acquainted and up-to-date with this SOP.

5. PROCEDURES:

5.1. Inclusion Criteria

- 5.1.1. The *plasmodium falciparum* is in category 6.2 ‘ Infectious substance, affecting humans’
- 5.1.2. Wear appropriate protective clothing the spill should be handled IAW IATA rules on shipment of infectious substances.
- 5.1.3. The potential donors will be aged between 18 years and 50 years inclusive.
- 5.1.4. The potential donors will be seen to be generally in good health as determined by the clinician following a physical examination and feeling well.
- 5.1.5. Willing to give informed consent.

5.2. Exclusion Criteria

- 5.2.1. Blood pressure that is out of the normal range.
- 5.2.2. Hemoglobin levels that are below acceptable levels.
- 5.2.3. Chronic illness.
- 5.2.4. Presence of any lesions or scars of needle pricks at the venipuncture site that is indicative of addiction to narcotics and/or frequent blood donation.
- 5.2.5. Frequent travel to malaria endemic areas – more than once a month for most months in a year
- 5.2.6. Prior prolonged residence in a malaria endemic area – more than 3 years

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- 5.2.7. Frequent malaria episodes in the past – more than once a month for most months in a year
- 5.2.8. Pregnancy
- 5.2.9. Lactating mothers
- 5.2.10. Findings that in the opinion of the investigator may result in adverse outcomes should the volunteer continue participation
- 5.2.11. Donation of blood within the last 6 months,
- 5.2.12. Taking antimalarial drugs or antibiotics within the last 2 weeks.

5.3. Identification of donor

- 5.3.1. Blood group determination, malaria blood film and blood count will be done
- 5.3.2. Testing of HIV and Hepatitis and other blood borne pathogens **WILL NOT** be conducted but volunteers will be encouraged to test.
- 5.3.3. Clinicians will ensure HB levels are within normal range(14-18g/dl for male and 12-16 g/dl for females)
- 5.3.4. No malaria parasite should be detected in blood
- 5.3.5. Volunteer should not have donated blood within the preceding 6 months or taken antibiotics within the preceding 2 weeks
- 5.3.6. Volunteers will be requested either to donate blood that will be used for the preparation of red blood cells or to donate blood for the preparation of serum. Volunteers will not donate blood for both purposes at the same blood draw

5.4. Informed consenting See attachment 3

5.5. Pretests

- 5.5.1. Malaria blood film
- 5.5.2. Complete blood count
- 5.5.3. Blood grouping

5.6. Post donation

- 5.6.1. Vitals signs are taken one hour after blood donation and you check the following:
- 5.6.2. Blood pressure
- 5.6.3. Auxiliary temperature
- 5.6.4. Pulse
- 5.6.5. After all the checks and the client are in good condition he /she is free to go home.

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- 5.6.6. In case of a problem the nurse will indicate plan of action in the progress notes

5.7. Volume of blood

5.7.1. The volume of blood collected should be 288 units of 400mls each and 144 units of 60mls each will be required per year.

5.7.2. Institutional SOP will be used for blood collection KSM/MDR_4819.

5.8. Blood transportation

5.8.1. Blood collected from the volunteers will be shipped at a temperature of between 2⁰C to 8°C to the Malaria Drug Resistance laboratory located in Kisumu by courier

5.8.2. The blood need to reach the lab within 24 hours of blood collection

5.8.3. The blood samples need to be packed according to the **IATA** rules.

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6. REFERENCES:

Reference Number or Author(s)	Document Title
SOP #	Phlebotomy KSM/MDR_4819
NCCLS Publication, H3-A7	Technique for Venous Puncture, 1987
<i>Science</i> Trager, W. and Jensen, J.B. (1976)	Human malaria parasites in continuous culture. <i>Science</i> , 193: 673 -675
WRAIR 1919 Version 01	Human blood collection for the in vitro culture of malaria parasites

7. FORMS AND APPENDICES:

Form or Attachment Number	Title
Attachment 1	SOP Read and Review Signature Log
Attachment 2	SOP Training Sign In Log
Attachment 3	Informed consent log
Attachment 4	Inclusion and Exclusion criteria log
Attachment 5	Laboratory results log
Attachment 6	Post donation log
Attachment 7	Risk Assessment Form

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8. DOCUMENT REVISION HISTORY:

SOP No.	Title	Version	Brief Description of Change	Effective Date
GEI 397	Standard operating procedure for Blood collection using protocol # WRAIR 1919	00	SOP Number assignment, SOP Training Requirements, Document Copy Control was updated. Approving Authority was also changed, Changed Logo, signatory section, Risk Assessment Form	20 th AUGUST 2013
KSM/MDR_4615	Standard operating procedure for Blood collection using protocol # WRAIR 1919	01	Changes in the version of the protocol and the WRAIR #	02-07-2018
KSM/MDR_4615	Standard operating procedure for Blood collection using protocol # WRAIR 1919	02	Current	Refer to Stamp

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9. DOCUMENT COPY CONTROL

SOP Distribution for: Standard operating procedure for Blood collection using protocol # WRAIR 1919	
ORIGINAL:	RA Office
COPY 1	Culture laboratory
2	MDR Molecular laboratory 1
3	MDR Molecular laboratory 1
4	DEID Sentinel Sites
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Attachment 1: SOP Read and Review Signature Log

By signing this Log, I confirm that I have read and understood the content of this SOP and I am comfortable applying them in my day to day operations as applicable

Printed Name	Title	Signature	Date
1.			
2.			
3.			
4.			
5.			
6.			
7.			

Provider's Name: _____ Signature: _____

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Attachment 2: SOP Training Sign In Log

By signing this Log, I confirm that I attended SOP training and understood the content of this SOP and I am comfortable applying them in my day to day operations as applicable.

SOP Training Date: _____

Printed Name	Title	Signature
1.		
2.		
3.		
4.		
5.		
6.		
7.		

8.		
----	--	--

Provider's Name: _____

Signature: _____

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INFORMED CONSENT

Has the subject provided the written informed consent? Yes No

If yes, Informed Consent Date and time: |_|_| |_|_| |_|_| |_| Time: |_|:|_|/|_|/|_| hr
dd mmm yyyy

If No, exclude

Has a copy of signed informed consent been given to the subject?

Yes No If No explain _____

DEMOGRAPHIC DATA.

Subject's initials I_|_|_|_| Gender: Male Female Subject's Age: _____
 Years

Area of Residence:
 Town: _____ (If available)

VITAL SIGNS AND ANTHROPOMETRY

Axillary Temperature: |_|_|.|_| °C
 Pulse: |_|_|_| b/min

Blood Pressure: |_|_|_|/|_|_|_| mmHg
Sys Dias

Weight: |_|_|.|_| kg

Vitals Taken by: ___ Date: |_|_| |_|_|_|_|_| Time: |_|_|/|_|_|/|_|_|
Initials dd mmm yyyy 24hr

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PHYSICAL EXAMINATION

Perform a complete physical examination and record the findings in the section below.

STEM	Normal	Abnormal	If abnormal, please specify	Check box if not done
Respiratory	<input type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
Cardiovascular	<input type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
Neurological	<input type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
DENT	<input type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
Gastrointestinal	<input type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
Reproductive	<input type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
Musculoskeletal	<input type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
Dermatological	<input type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
Immunological/ Lymphatic	<input type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

Attachment 4- Inclusion and Exclusion criteria Log for S.O.P File.

All must be **YES** for **INCLUSION**

	YES	NO
Adult aged between 18 years and 50 years inclusive	<input type="checkbox"/>	<input type="checkbox"/>
Generally in good health and feeling well	<input type="checkbox"/>	<input type="checkbox"/>

EXCLUSION CRITERIA

All must be **NO** for inclusion

	YES	NO
Blood pressure that is out of range.	<input type="checkbox"/>	<input type="checkbox"/>
Hemoglobin levels that are below acceptable levels	<input type="checkbox"/>	<input type="checkbox"/>
Chronic illness	<input type="checkbox"/>	<input type="checkbox"/>
Presence of any lesion or scar of needle pricks at the venipuncture site that are indicative of addiction to narcotics and/or frequent blood donation.	<input type="checkbox"/>	<input type="checkbox"/>
Frequent travel to malaria endemic areas	<input type="checkbox"/>	<input type="checkbox"/>
Prior prolonged residence in a malaria endemic area	<input type="checkbox"/>	<input type="checkbox"/>
Frequent malaria episodes in the past	<input type="checkbox"/>	<input type="checkbox"/>
Pregnancy	<input type="checkbox"/>	<input type="checkbox"/>
Lactating mothers	<input type="checkbox"/>	<input type="checkbox"/>
Findings that in the opinion of the investigator may result in adverse outcomes should the volunteer continue participation	<input type="checkbox"/>	<input type="checkbox"/>
Donation of blood within the last 6 months	<input type="checkbox"/>	<input type="checkbox"/>
Taking antimalarial drugs or antibiotics within the last 2 weeks	<input type="checkbox"/>	<input type="checkbox"/>

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Attachment 5- Laboratory Results Log

LABORATORY TESTS RESULTS

Blood group _____

Hemoglobin |__|__|.____| g/dl

Hemoglobin within acceptable range? YES NO

Malaria blood film negative? YES NO

*If any of the above is NO, the volunteer is **not** eligible to donate blood.*

Appendix 5 Standard Operating Procedure for Optimization of GIA assay

US Army Medical Research Unit – Kenya

GEIS PROJECT

Standard Operating Procedure

SOP TITLE: : Testing <i>In vitro</i> Plasmodium falciparum parasite growth inhibition using immune and non immune serum	SOP No. 001	GEI
	Version	.05
Effective date 25 TH March 2015	Page	1 of 8

Signatures and Dates:

Author: ----- Date -----
CHRISTINE WANJALA

QA Review: ----- Date -----

Approving

Authority: ----- Date -----
MAJ. EDWIN KAMAU

Review / Approval for unchanged documents

Author	QA Review	Approving Authority

1 PURPOSE / APPLICABILITY

1.1 Purpose: To provide procedures for testing of *Plasmodium falciparum* parasite growth inhibition by immune and non immune serum

1.2 Applicability: This SOP applies to immunology assay carried out in WRP Kisumu MDR Laboratory personnel, Laboratory manager, trainees, principal investigator (PI)

2 ABBREVIATION AND TERMS:

- 2.1 CMS -Complete medium
- 2.2 MI -Millilitres
- 2.3 RBC -Red blood cells
- 2.4 RPM -Revolution per minute
- 2.5 RPMI 1640 -Roswell Park Memorial Institute 1640 cell culture medium
- 2.6 RT -Room temp
- 2.7 SOP -Standard Operating Procedures
- 2.8 ul -Microlitre
- 2.9 SLS -Senior lab supervisor
- 2.10 Hrs -Hours

3 EQUIPMENTS AND MATERIALS:

3.1 Equipments

- 3.1.1 Centrifuge
- 3.1.2 Medical grade gas N₂, 90% CO₂, 5% O₂, 5%

3.2 Materials

- 3.2.1 Serological pipette
- 3.2.2 Filter units
- 3.2.3 Zip-lock bag

3.3 Reagents

- 3.3.1 Distilled water
- 3.3.2 Phosphate buffered saline
- 3.3.4 Non immune serum
- 3.3.5 Immune serum
- 3.3.6 Red blood cells
- 3.3.7 SYBR Green I
- 3.3.8 Lysis buffer
- 3.3.9 10% complete medium
- 3.3.10 Ethanol
- 3.3.11 Glucose
- 3.3.12 Hepes
- 3.3.13 Sodium bicarbonate
- 3.3.14 3D7 Parasites
- 3.3.14 RPMI basic medium
- 3.3.15 Giemsa stain

4 RESPONSIBILITIES:

- 4.1 The SLS will ensure that these procedures are strictly adhered to and that all Personnel required to perform these procedures are properly trained
- 4.2 It is the responsibility of the lab director and lab manager to ensure that this document is up to date.
- 4.3 It is the responsibility of the technical personnel to understand and adhere to this SOP

5 PROCEDURES:

5.1 Preparation of reagents

5.1.1 Immune serum

- 5.1.1.1 Using 5ml serological pipette transfer patient's serum into 15ml centrifuge tube and centrifuge at 2500 RPM for 3 min
- 5.1.1.2 Aliquot the serum layer into a clean well labelled vial for use as immune serum
The remaining sample is washed using wash medium then cultured and cry preserved.

5.1.2 Non immune serum

- 5.1.2.1 Thaw the non immune serum (**O+ ve**) in 37 °C water bath prior to use
- 5.1.2.2 Remove non immune serum from water to room temperature (RT) 20 °C - 23 °C as soon as thawed (about 30 min)

5.1.3 3D7 Parasites

- 5.1.3.1 Check the percentage parasitemia using thin smear prior to setting the assay
- 5.1.3.2 The percentage parasitemia should be diluted to 1 % at 2 % haematocrit using 50 % washed Red blood cells.

5.1.4 MSF Lysis buffer containing SYBR Green I

- 5.1.4.1 Thaw one SYBR Green I aliquot vial in the incubator for 5 min
- 5.1.4.2 Add 4 µl of SYBR Green I to 2 mL of MSF lysis buffer. The solution is adequate for 12 wells of immune and non-immune serum in duplicates
- 5.1.4.3 Pipette to mix, avoiding the production of bubbles (This solution is made fresh prior to assay harvest)

5.1.5 RPMI basic medium (1L)

- 5.1.5.1 Add 1 packet of powdered medium in 850ml of distilled water in a cylinder with magnetic stirrer on a magnetic bar
- 5.1.5.2 Add 2 g of glucose
- 5.1.5.3 Add 5.95 g Hepes
- 5.1.5.4 Stir at medium speed until completely dissolved
- 5.1.5.5 Filter using 0.2 μM filter unit label and indicate the preparation and expiry date then store in 2-8°C, good for 30 days

5.1.6 serum-free media

- 5.1.6.1 Add 1.0 mL 1.45 mM sterile of hypoxanthine to 43.4mL of RPMI basic medium
- 5.1.6.2 Add 1.6 mL of sterile sodium bicarbonate, 7.5 %
- 5.1.6.2 Filter with 0.2 μM then Store at 2-8°C. Good for 2 weeks

5.1.7 Complete medium

- 5.1.7.1 **10% CMS**
Add 5 mL of serum to 43.4 mL of RPMI basic medium
Add 1.0 mL 1.45 mM sterile hypoxanthine
Add 1.6 mL of sterile sodium bicarbonate, 7.5 %
Filter with 0.8 μM , 0.45 μM and 0.2 μM then
Store at 2-8°C. Good for 2 weeks

5.1.8 Preparation of 5% sorbitol solution

- 5.1.8.1 Add 50 g of D-sorbitol to 1 L of culture water.
- 5.1.8.2 Stir until completely dissolved.
- 5.1.8.4 Sterilize the solution using a 0.2 μM pore size, 1 L filter.
- 5.1.8.4 Store at 4°C up to 1 year

5.2 Technical procedure

- 5.2.1 In a Bio-safety cabinet, wiped and cleaned with 70% ethanol
- 5.2.2 Place 48 well plates in the bio-safety cabinet and labelled the plate for immune and non-immune serum at 10 folds difference concentration of serum to avoid confusion, Note that the well containing 10 % CMS will not have serum.
- 5.2.3 Pipette 500 μl of 10 % CMS in the first two well, which are the positive control
- 5.2.4 Using serum-free media, prepare 10 % vol/vol CMS using immune serum (0.5 mL immune serum +4.5 mL serum-free media) as the immune CMS. Immune serum also represents all test sera from malaria patients
- 5.2.5 In a separate test tube, use serum-free media to prepare 10 % vol/vol CMS using non-immune serum (0.5mL non-immune serum +4.5 mL serum-free media) as the non-immune CMS.
- 5.2.6 To prepare 10-fold dilution of the immune sera, add 0.5 mL of the immune CMS to 4.5 mL non-immune CMS.
- 5.2.7 To prepare 10-fold dilution of the non-immune sera, add 0.5 mL of the non-immune CMS to 4.5 mL non-immune CMS
- 5.2.8 Add of these CMS to the wells as indicated in the plate map under appendix
- 5.2.9 To test the *in vitro* activities, add 10 μL of 3D7 parasite in each well diluted to 1 % parasitemia at 2 % hematocrit
- 5.2.10 Mix each well gentle to avoid bubbles
- 5.2.11 Repeat the procedure using non-immune serum in duplicates on the same plate as described above then flash it with 90 % N_2 , 5 % O_2 , and 5 % CO_2 Gas mixture
- 5.2.12 Indicate the date of termination and incubate at 37 °C for 72 hrs.

5.3 Termination

- 5.3.1 In a Bio-safety cabinet, wiped and cleaned with 70 % ethanol
- 5.3.2 Gentle mix each well using pipette to have uniform mixture after incubation (72 hrs)
- 5.3.3 Aliquot each well labelled appropriately into respective vials

5.3.4 In 96 well plate labelled in accordance to the set up (Refer to appendix 2 for 96 plate set up template)

5.3.5 Pipette 100 µl of the mixture from each vial to their respective wells labelled (Refer to appendix 2 for 96 plate set up template)

5.3.6 Add 100 µl of SYBR Green I with lysis buffer in each well. This process is done away from light

5.4.7 The remaining mixture in the vials, aliquot 200 µl in another clean vial, centrifuge and prepare thin smears across all concentrations in immune and non immune

5.4.8 Store the remaining mixture in their respective vial in -80 °C

5.4 Reading the plate and interpreting results

5.4.1 Insert the 96 well plates in Tecan reader then read the plate using the reader and interpret.

Well plate set up

IMMUNE SERUM			NON IMMUNE SERUM			
Positive control	10% imm-cms	1% imm-cms	Positive control	10% Non-Imm cms	1% Non-Imm cms	
Positive control	10% imm-cms	1% imm-cms	Positive control	10% Non-Imm cms	1% Non-Imm cms	

10%cms	10% cms	10% cms	pos ctl
10% imm-cms	10% imm-cms	10% imm-cms	Imm serum
1%imm-cms	1%imm-cms	1%imm-cms	
10%Non-Imm cms	10%Non-Imm cms	10%Non-Imm cms	
1% Non- imm cms	1% Non- imm cms	1% Non -imm cms	

6 References:

Reference number or Authors	Document Title.
WON-001	Procedure s and format for SOP and related documents

7 Forms and Appendices:

Forms or Appendix Number	Title
APPENDIX 1	48 well plate set up.

8 Document Revision History:

Version Number	Description of changes	Effective Date
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Original; Title : Testing <i>In vitro</i> <i>Plasmodium falciparum</i> parasite growth inhibition using immune and non immune serum	Original	March 2015
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Appendix 6. Study sample collection procedures for adults

Study procedures	Priority	Assay Volume	D0 ¹	D1	D2	D3	D4	D7	D14	D21	D28	D35	D42	RC ⁶
Eligibility Evaluation			-1											
Informed Consent/Assent			-1											
Demographics			-1											
Pregnancy test (from urine)			-1											
Physical Examination ²			X	X	X	X	X	X	X	X	X	X	X	X
Adverse Events			X	X	X	X	X	X	X	X	X	X	X	X
Vital Signs			-1, X	X	X	X	X	X	X	X	X	X	X	X
Concomitant Medications			X	X	X	X	X	X	X	X	X	X	X	X
Urine sample collection			X											
Artesunate (mg/kg)*			4	4	4									
Mefloquine (mg/kg)*						15	10							
Artemether-Lumefantrine*			X	X	X									
Blood smears ¹	1	0.1ml	-1, 0, 4,	24, 30,	48, 54,	72,Δ	Δ	X	X	X	X	X	X	X
PCR for speciation	1	1ml	-1											X
Real-time PCR	1	0.8ml	0, 4, 8,	24, 30,	48, 54,	72,Δ	Δ	X	X	X	X	X	X	
Hematology ⁴	1	1ml	X	X	X	X		X	X	X	X	X	X	X
Pharmacokinetics	2	2ml	X			X			X					X
In vitro susceptibility testing	2	3ml	X											X
Cryopreservation	3	2ml	X											X
Storage in RNAlater (PTEP)	3	1ml	X											X
Microsatellite Markers, SNPS	3	2ml	X											X
Reference Molecular GWAS	3	3ml	X											X
Serology	2	5ml	X						X				X	X
PBMCs ⁵	3	1ml	X											X
Blood Clotting	3	4ml	X											
Cytokine Determination	3	2ml	X											
Biochemistry (glucose)**	1	1ml	X			X								
Bioassay	2	2ml	X											X
Total blood collected			34.6	4.6	4.6	7.6	-	1.9	8.9	1.9	1.9	1.9	6.9	23.1

¹ Numbers show hour of blood sampling for microscopy, -1 is pre-enrollment; "X" indicates one sampling per day, "Δ" indicates that sample will be collected q6 until two consecutive samples are negative by microscopy

² Complete physical exam on Day 0, Targeted physical on all subsequent days

³ Biochemistry tests include glucose, creatinine, AST, ALT.

⁴ Hematology tests include Hb, hematocrit and platelets

⁵ PBMCs = Peripheral blood mononuclear cells

⁶ RC = recrudescence within the 42 days of follow-up

⁷ Total blood volume collected for the entire study = 74.8mL. For participants with recrudescence, total volumes will be 97.9 mL.

* Depending on randomization scheme. 1st MQ dose is supervised, 2nd dose is unsupervised. ** - if needed

Appendix 7: Study Sample collection procedures for children

Study procedures	Priority	Assay Volume	D0 ¹	D1	D2	D3	D4	D7	D14	D21	D28	D35	D42	RC ⁶
Eligibility Evaluation			-1											
Informed Consent/Assent			-1											
Demographics			-1											
Pregnancy test (from urine)			-1											
Physical Examination ²			X	X	X	X	X	X	X	X	X	X	X	X
Adverse Events			X	X	X	X	X	X	X	X	X	X	X	X
Vital Signs			-1, X	X	X	X	X	X	X	X	X	X	X	X
Concomitant Medications			X	X	X	X	X	X	X	X	X	X	X	X
Artesunate (mg/Kg)			4	4	4									
Mefloquine* (mg/Kg)						15	10							
Artemether-Lumefantrine*			X	X	X									
Urine sample collection			X											
Blood smears ¹	1	0.1ml	-1, 0, 4,	24, 30,	48, 54,	72,Δ	Δ	X	X	X	X	X	X	X
PCR for speciation	1	1ml	-1											X
Real-time PCR	1	0.4ml	0, 4, 8,	24, 30,	48, 54,	72,Δ	Δ	X	X	X	X	X	X	
Hematology ⁴	1	0.4ml	X	X	X	X		X	X	X	X	X	X	X
Pharmacokinetics	2	2ml	X			X			X					X
In vitro susceptibility testing	2	2ml	X											X
Cryopreservation	3	1ml	X											X
Microsatellite Markers, SNPs	2	2ml	X											X
Reference Molecular GWAS	3	2 ml	X											X
Serology	2	2ml	X						X				X	X
PBMCs ⁵	3	0.5ml	X											X
Blood Clotting	3	2ml	X											
Cytokine Determination	3	1ml	X											
Biochemistry (glucose)**	1	1ml	X			X								
Bioassays	2	2ml	X											X
Total blood collected (mL)⁶			21.5	2.4	2.4	5.4	-	0.9	4.9	0.9	0.9	0.9	2.9	15

¹ Numbers show hour of blood sampling for microscopy, -1 is pre-enrollment; “X” indicates one sampling per day; “Δ” indicates that sample will be collected q6 until two consecutive samples are negative by microscopy.

² Complete physical exam on Day 0, Targeted physical on all subsequent days

³ Biochemistry tests include glucose, creatinine, AST, ALT.

⁴ Hematology tests include Hb, hematocrit and platelets

⁵ PBMCs = Peripheral blood mononuclear cells

⁶ RC = recrudescence within the 42 days of follow-up

⁷ Total blood volume collected for the entire study = 43.1mL. For participants with recrudescence, total volumes will be 58.1mL

*Depending on randomization scheme. 1st MQ dose is supervised, 2nd dose is unsupervised. ** if indicated