DETECTION OF SEXUAL STAGE PARASITES IN ANOPHELES MID GUT POST MEMBRANE FED WITH *PLASMODIUM FALCIPARUM* GAMETOCYTES

MASTER OF SCIENCE IN ENVIRONMENTAL HEALTH THESIS

ELLEN MARTHA SITHEMBILE NDHLOVU

UNIVERSITY OF MALAWI

THE POLYTECHNIC

April, 2018

DETECTION OF SEXUAL STAGE PARASITES IN ANOPHELES MID GUT POST MEMBRANE FED WITH *PLASMODIUM FALCIPARUM* GAMETOCYTES

MASTER OF SCIENCE IN ENVIRONMENTAL HEALTH THESIS

By

ELLEN MARTHA SITHEMBILE NDHLOVU
BSc.E.H. (University of Malawi)

UNIVERSITY OF MALAWI

THE POLYTECHNIC

April, 2018

DECLARATION

I declare that this research entitled 'Detection of sexual stage parasites in Anopheles mid gut post membrane fed with *Plasmodium falciparum* gametocytes" is my work. It is submitted in partial fulfilment of the requirements for the Master of Science Degree in Environmental Health at the Polytechnic, University of Malawi. It has not been submitted for any other degree to any University.

Signed:

Candidate's Full Name: Ellen Martha Sithembile Ndhlovu

CERTIFICATE OF APPROVAL

We, the undersigned, certify that we have read and hereby recommend for acceptance by University of Malawi, The Polytechnic, a thesis entitled '*Detection of sexual stage parasites in Anopheles mid gut post membrane fed with Plasmodium falciparum gametocytes*.

Dean of Postgraduate : _____

Signature : _____

Date : _____

Main Supervisor : _____

Signature : _____

Date : _____

Co-supervisor : _____

Signature : _____

Date :_____

Head of Department : _____

Signature : _____

Date : _____

DEDICATION

To my late mum, Christina Hunga and dad, Mr Joseph Ndhlovu.

You went too early; I wish you lingered a bit.

To my son, DUDLEY WONDERFUL

This is for you. We came a long way.

ACKNOWLEDGEMENTS

Glory and honour to the living God Almighty, for his mercies shall endure forever, ever faithful, ever true. Thank you Father for proving to me that when you are involved in my case, it does not matter who else is involved.

Special appreciation to my supervisors, Themba Mzilahowa, Rex Mbewe and Kingsley Lungu for the technical support and guidance all through. I do not know what I could have done without your presence.

Very special thanks to my only son Dudley Wonderful for the love, patience and understanding throughout. I was away for long when you needed me most. We came along way and God has been faithful. Be assured of my total love and care. Through it all, we have learnt to depend on God the more.

I am greatly humbled by the love, prayers and support from my siblings Griffin, Joyce and Tiwonge. I would not trade you guys for anything in this world. We came a long way and God has seen us through.

I salute all my colleagues and friends so numerous to mention especially at Malaria Alert Centre (Blantyre) for the support both moral and academic. You will always find favour and peace in the eyes of the Lord. I cherish your love and support. God, bless you all.

ABSTRACT

The study was conducted to detect the sexual stage parasites (oocysts) of *Plasmodium falciparum* (*Pf*) in mosquitoes post membrane feeding. The main aim was to investigate the sexual development of laboratory cultured(*Pf*) gametocytes in *Anopheles* host using membrane feeding with the ultimate goal of understanding malaria transmission. Specifically, the focus was to determine optimal *f*eeding density and feeding duration, assessing infection success and infection rate and identifying bottlenecks to successful *Pf* gametocytes transfer through membrane feeding.

Mosquitoes were reared in the laboratory to produce F1 generation which is parasite free and later were inoculated with cultured *Pf* gametocytes (stage V). The gametocytes were fed to the mosquitoes through membrane feeding. Mosquito midguts were later dissected to detect the sexual stage parasites (oocysts) using microscopy after incubation period of 9 days.

Mosquito rearing and parasite culturing were successful. Successful parasite inoculation was confirmed by presence of gametocytes in the mosquitoes within 24 hours post feeding. However, oocysts were not detected in the mid gut after 9 days.

The study results showed that *P. falciparum* infection into the *Anopheles* was not complete as shown by failure to detect oocysts on the midgut and sporozoites on the salivary glands. This could be due to several factors such as but not limited to parasite maturity, parasite acquisition to its host, temperature maintenance and transmission efficiency,

The study concluded that gametocytes detection in the midgut has to be meticulously conducted to care for other factors that affect the viability of the parasites. However, the study successfully demonstrated the possibility of inoculating *Pf* gametocytes into mosquitoes through membrane feeding.

It is recommended that the three novel approaches; *in vitro* parasite culturing of *Plasmodium falciparum* gametocytes, rearing of *Anopheles* vector and membrane feeding, being the most critical procedures for the success of the study, have to be done according to the stipulated standards of operating procedures so that required results are yielded.

Table of Contents

LIST OF FIGURES.

LIST OF TABLES

LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy
COM	College of Medicine
CS	Circumsporozoite
DNA	Deoxyribonucleic Acid
EIP	Extrinsic Incubation Period
GlcNAc	*N*-acetylglucosamine
HIV	Human Immunodeficiency Virus
ICEMR	International Centre of Excellence in Malaria Research
LDH	Lactate dehydrogenase
MAC	Malaria Alert Centre
NMCP	National Malaria Control Programme
PCR	Polymerase Chain Reactions
Pf	*Plasmodium falciparum*
PPE	Personal Protective Clothing
QA	Quality Assurance
QC	Quality Control
RT	Real-Time
RBC	Red Blood Cells
SOP	Standard Operating Procedures
TB	Tuberculosis

APPENDICES

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information.

Malaria continues to be one of the world's most devastating diseases. It is caused by a protozoan parasite of the genus *Plasmodium*. Different from other major infectious diseases like Human Immunodefiency Virus (HIV) and Tuberculosis (TB), the malaria parasite requires a mosquito vector for transmission to occur. Though mosquitoes are vectors of many fatal and debilitating diseases, none is more challenging than malaria. Malaria incidence and burden may be much higher than what is actually reported. This presents distinct challenges as interventions shift from malaria control to elimination in endemic settings.

In this era of environmental change, resurgent diseases and insecticide resistance, the need to explore alternative and novel approaches is urgent but requires an intimate understanding of parasite-to- host interactions. Logically, any control intervention should target the parasite at the most vulnerable stage of its life cycle. It has to be noted that the *Plasmodium* parasite undergoes sexual reproduction once during its lifecycle and this only occurs in a mosquito vector. Within the *Anopheles* mosquitoes, the only natural vectors of human malaria, these stages occur mostly dramatically at two epithelial interfaces-the mid gut and the salivary gland, both of which represent major survival bottlenecks for the parasite (Whitten et al., 2006).This, therefore makes the sexual stages of the parasite in a mosquito important targets for the control of malaria transmission. The malaria parasite lifecycle constitutes one of the most complicated and fascinating of any organisms and thus poses intriguing areas of study for cell biology, molecular biology as well as immunology.

Parasite development in its vector starts when a mosquito ingests an infected blood meal containing *Plasmodium* sexual forms known as gametocytes. In the case of *Plasmodium*

falciparum (*Pf*), within 15 minutes, gametocytes round up, egress from the red blood cells and differentiate into gametes. Male gametocytes undergo a drastic transformation known as exflagellation to form microgametes. Microgametes detach from the exflagellation centre and actively search for female gametes to fertilise. Fertilisation results into zygotes that subsequently differentiate into motile ookinetes in the mid gut lumen.

Notably the ookinete is the only invasive stage that is not preceded by a replication step and thus ookinete numbers are a direct product of the number of fertilisation events(Aly et al., 2009).The ookinete starts its short journey by transversing the mid gut epithelial cell layer from the apical cells and then egresses from the basal end to reach the basal lamina. Sometimes this invasion step is accompanied by a severe reduction in the ookinete numbers due to the host protective mechanisms. The surviving ookinetes become sessile and transform to oocysts which is the only parasite developmental stage that grows extracellulary and within 10-14 days each oocyst grows in size and undergoes sporogony to produce (through mitosis) thousands of sporozoites which is the infective stage of the malaria parasite and is destined to infect the mammalian liver. Sporozoites acquire motility before they get released from the oocysts (Smith et al., 2014). Migration is through several hepatocytes before infecting the final one and this occurs by breaching their plasma membranes. When Sporozoites are released in the body cavity of the mosquito and invade salivary glands, they may suffer severe loses on this journey thus the ookinetes and sporozoites are the bottle neck stages in the malaria parasite lifecycle (Vlachou, 2006).

In summary, the developmental processes of a *Plasmodium* parasite in the mosquito (which is shown in Figure 2 in the next session) are: 1) gametogenesis and ookinete formation, 2) ookinete invasion of the midgut epithelium, 3) ookinete to oocyst transformation, 4) oocyst development and sporozoite differentiation, 5) sporozoite maturation and 6) development of infectivity which involves egress of sporozoites into mosquito hemocoal as well as attachment and invasion of the salivary glands (Aly et al., 2009).

1.2 Rationale/Justification of the study

As the fight against malaria continues, there is need to understand both human and parasite factors that govern disease transmission. More importantly the knowledge of who transmits and parasite attenuation as it passes through the vector host are some of urgent and critical steps.

Recently there has been increased investment into the fight against malaria in Malawi but little seems to happen to reduce the burden of the disease. It is therefore important that studies on parasites and their transmission dynamics are undertaken. Since malaria parasites must complete a complex developmental cycle in *Anopheles* mosquito vector before transmission to a vertebrate host, this makes the mosquito an obligatory vector hence interference with parasite development whilst in it will result in decreased transmission.

While details of the host- parasite co-evolution are only beginning to emerge, this study also highlighted the ability of the parasite to adapt to its vector host to ensure survival at each stage of development since intrinsic vector characteristics and environmental factors has proved to affect the sporogonic development of plasmodium falciparum in *Anopheles* mosquitoes (Douglas et al., 2015).

The bottle neck in malaria parasite numbers within its mosquito host argues that the mid gut (where sexual reproduction occurs) is an optimal target for intervention strategies to block malaria transmission and prevent infection hence this study aimed to fully understand the plasmodium parasite migration in the mid gut of a mosquito vector so that proper and sound prevention measures can be developed in trying to control malaria infection.

Not only was the presence of the appropriate life cycle in the mosquito mid gut (gametocyte) ably tested, but also the development of the parasite from the gametocyte to the oocyst and subsequently to a sporozoite was ably tracked – all developmental progressions that were needed for the progression of an infection from the bite of an infected individual to the generation of an infectious mosquito.

1.3 Problem Statement

Initial studies have been conducted which target the parasite development at the most vulnerable stage of its lifecycle to block malaria transmission. One such study was conducted by Bassiouni (2015) who worked on a project to attempt to determine the lower limit of detection of parasites within the mid gut of a mosquito. This is a carry forward project and is partially motivated by the expanded entomologic interest of the International Centres of Excellence in Malaria Research 1 (ICEMR 1), which will continue into ICEMR 2. The interest expands beyond the mere detection of parasites to include the ability to distinguish parasites of different genotypic background. The initial results were unexpected, in that the level of detection was an order of magnitude worse than that of parasites in the blood stream. Possible improvements that could increase the sensitivity include 1) the use of gametocytes in the membrane feed rather than asexual parasites and 2) the use of a gametocyte specific RT-qPCR rather than the LDH qPCR. The recent transfer of a technique for *in vitro* generation of gametocytes to the Molecular Core will allow this project to become much more physiologically relevant.

1.4 Objectives of the study

1.4.1 Broad Objective

To investigate the sexual development of laboratory cultured *Plasmodium falciparum* (*Pf*) gametocytes in *Anopheles* host using membrane feeding.

1.4.2 Specific Objectives

i. To determine the optimal *Anopheles* feeding density and feeding duration through membrane feeding.
ii. To assess infection success and *Pf* oocyst density (infection rate) in *An. gambiae* Kisumu midgut.
iii. To identify bottlenecks to successful *Pf* gametocytes, transfer through membrane feeding experiments.
iv. To compare *Pf* infection rates between *An. gambiae* Kisumu and wild caught *An. funestus sensu stricto*

CHAPTER TWO

2.0 LITERATURE REVIEW

Vector borne diseases constitute an enormous burden on public health across the world. However, despite the importance of interaction between infectious pathogens and their respective vector for disease transmission, the biology of the pathogen in the insect is often less well understood than the forms that cause human infections.

This chapter will discuss the available literature on the developmental progression of malaria sexual stage parasites in a mosquito as well as the factors that affect *Plasmodium falciparum* infection in the vector.

2.1 Optimal mosquito infectivity with *Plasmodium* using membrane feeding assays

Vallejo et al., (2016) researched on optimisation of a membrane feeding assay for *Plasmodium Vivax* in *Anopheles Albimanus.* The study involved assessment of parasite infectiousness by membrane feeding assay using laboratory reared *Anopheles* mosquitoes. Infection was measured by qPCR and by microscopically examining mosquito midguts at day 7 for presence of oocysts. Mosquito and parasite host factors that may affect the outcome of parasite transmission as measured by membrane feeding assays were also evaluated. Specifically, but not limited to, the optimal *Anopheles* feeding density, feeding duration as well as oocysts quantification methods were established.

Results concluded that optimal mosquito infectivity occurs with mosquitoes four days after emergency at a cage density of 100 and is best quantified by PCR as it may be underestimated by microscope. Further, host cellular immune response did not appear to significantly affect mosquito infectivity and no statistically significant difference was observed in transmission between mosquitoes directly feeding on humans and artificial membrane feeding assays.

2.2 *Plasmodium falciparum* migration through a mosquito mid gut

Malaria parasite migration through a mosquito host constitutes a major population bottleneck of the lifecycle and therefore represents a powerful, although as yet relatively untapped target for therapeutic interventions.

Meis et al., (1989) confirmed that the migration of ookinetes takes an intracellular route through the midgut epithelium. Evidence is present that oocyst capsule formation begins as early as during

the migration of ookinetes. After localisation between epithelial cells and the midgut basal lamina, the rapidly expanding oocyst stretches the overlying layer of the later at the heamocoele surface while a new basal lamina is generated between the oocyst and the epithelial cells.

Gouagna et al., (1998) tried to investigate the successive losses in the parasite densities of *Pf* stages during early sporogonic cycle in the laboratory, where reared *Anopheles gambiae* were infected through membrane feeding with blood from naturally infected gametocytes carriers. The developmental stages were studied from zygote to oocyst by immunoflourescent method using monoclonal antibodies against the *Pf* protein present on the surface of the newly formed gametes. This method allowed the assessment of the various sporogonic stages before, during and after the passage of the midgut wall. Parasite densities were determined within the entire blood meal at 3 hours (zygotes and macrogametes) and 24 hours (ookinetes) post infection, 48 hours after the mosquito blood meal, midguts were checked for the presence of early oocysts. For the mid-sized oocysts count, classic microscopy examination was used at day 7 of infection. The parasite efficacy was estimated by following successive losses in parasite densities between different early stages of the sporogonic in *Anopheles gambiae*.

2.3 Plasmodium – Anopheles mosquito interactions

Molecular strategies to study *Plasmodium-Anopheles* mosquito interactions by Ghosh et al.., (2003) revealed that it is a known factor that malaria kills millions of people every year but less well recognised is the fact that the situation is steadily deteriorating for a lack of effective means to counter step towards the development of new approaches to fight malaria as well as a thorough understanding of the mechanisms that direct parasite growth and differentiation including parasite host interactions. This supports the purpose of this study since its main aim is to understand the *Pf* migration in its mosquito host so that necessary measures are developed in an attempt to interrupt parasite transmission thereby controlling malaria infection

The malaria parasite has an absolute requirement for both a vertebrate host and a mosquito host in order to complete its life cycle and its interactions with the later provide the focus of this research. The mosquito mid gut represents one of the most challenging environments for the survival and development of the plasmodium and is thus also one of the most attractive for novel and targeted malaria control strategies.

Malaria parasites must complete a complex developmental cycle in an *Anopheles* mosquito vector before transmission to a vertebrate host. Sexual development of the parasite in the midgut is initiated in the lumen immediately after the mosquito ingests infected blood and the resulting ookinetes must transverse the surrounding epithelial layer before transforming to oocysts. The innate immune system of the mosquito is activated during midgut invasion which results in the losses of the parasite during development (Vega-Rodríguez et al., 2014)

2.4 Development of sexual *Pf* stages within a human host

The asexual stages of the malaria parasite are responsible for malaria transmission.Aly et al., (2009) discovered that in the human host, these asexual stages begin as sexually committed ring parasites, then develop into early stage gametocytes and finally mature into late stage gametocytes. Whereas only mature gametocytes are found in circulation, the immature stages are sequestered into vasculature and bone marrow before being released from sequestration into circulation. However, they only become infectious to the mosquito after they undergo a few days of additional maturation in circulation.

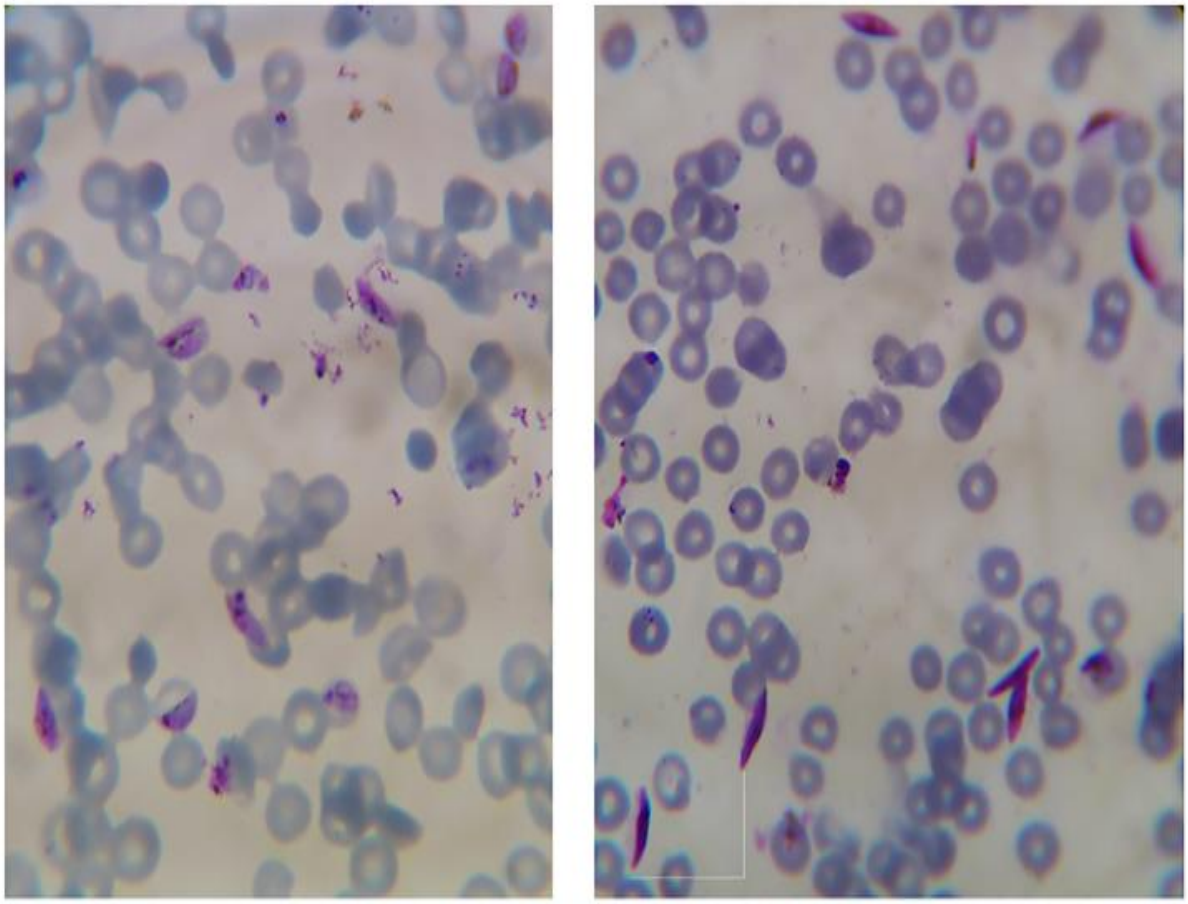

Figure 1: Development of Pf gametocytes sexual stages.

During a blood meal, the female *Anopheles* picks up mature stage gametocytes and these complete their sporogonic life cycle which results in the mosquito becoming infectious to humans as they harbour sporozoites within their salivary glands which are again passed onto a new human host during a subsequent blood meal of that infectious mosquito Amoah et al., (2015).

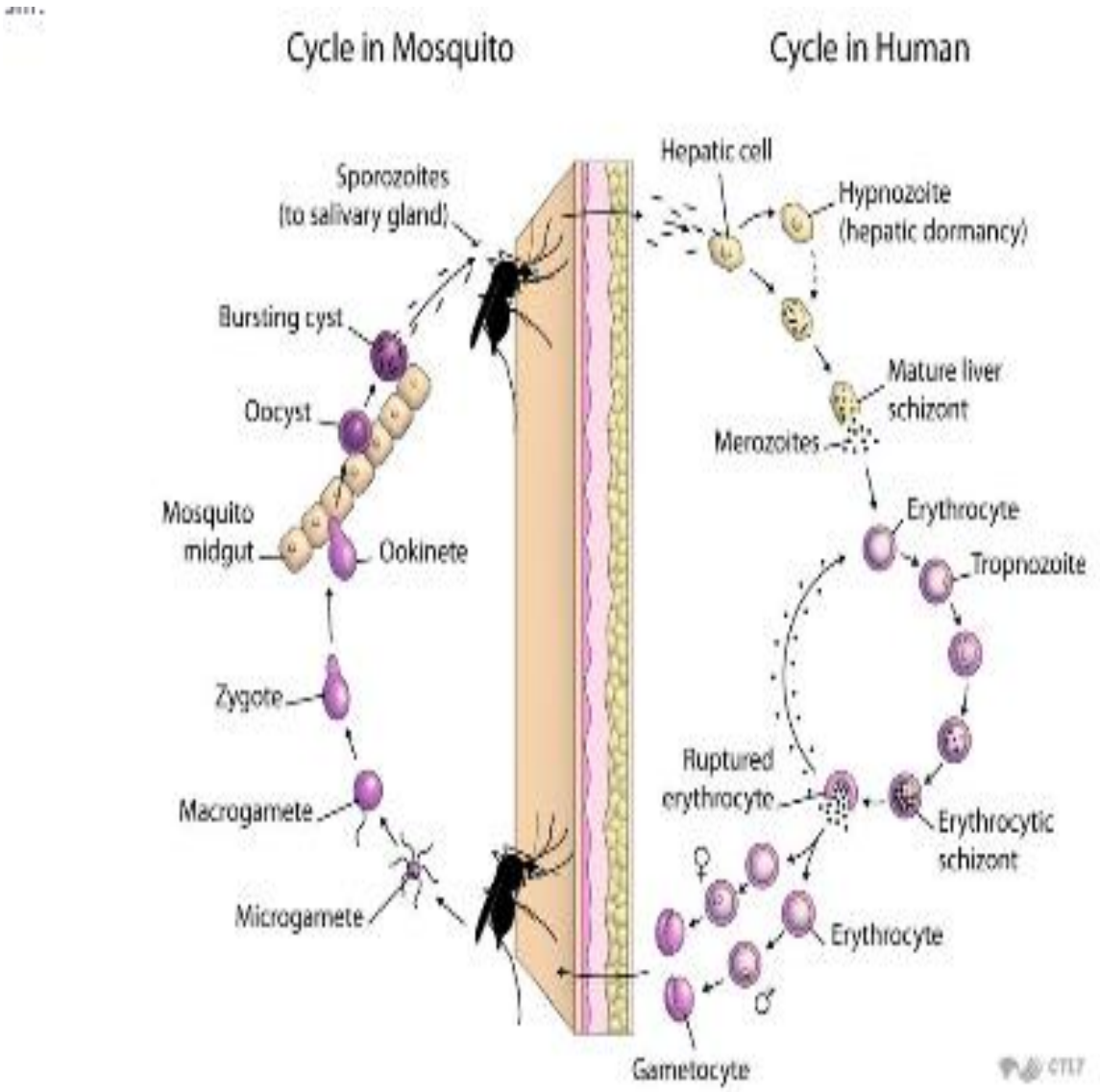

Figure 2: The life cycle Plasmodium falciparum parasite in both the vertebrate host and the Anopheles mosquito vector.

It is known that the pathophysiology of malaria is due to asexual stages of the *Plasmodium* parasite within the human host therefore anti-malarial drugs target the asexual stages with very few targeting the transmissible stages.

2.5 Transmission reducing interventions (TRI)

Plasmodium falciparum parasites are continuously evolving and have gained resistance to all orthodox antimalarial drugs including artemisinins. These coupled with the fact that there is currently no clinical vaccine for malaria, makes the discovery and production of new antimalarial drugs that target both the asexual and sexual stages of the parasite imperative.

The use of artemisinin and its derivatives from the herbal plant *Artemesia annua* in the last decade has increased interest in the potential of locally grown plants to provide new and more potent drugs in the treatment and eradication of malaria. Although artemisinin and its analogues have proven to be a wonder drug for the treatment of chloroquine resistant malaria, they are effective against the asexual parasites and only immature gametocytes thus there is still a high proportion of malaria patients harbouring highly infectious mature gametocytes hence an urgent need to identify antimalarial drugs with gametocytal activity to aid in malaria elimination agenda (Amoah et al., 2015).

The evaluation of transmission reducing interventions (TRI) to control malaria widely uses membrane feeding assays. In such assays, the intensity of *Plasmodium* infection in the vector might affect the measured efficacy of the candidates to block transmission.

Gametocytes density in the host blood is a determinant of the infection success in the mosquito, however, uncertain estimates of the parasite densities and intrinsic characteristics of the infected blood can induce variability. To reduce this variation, a feasible method is to dilute infected blood samples .The effect of diluting blood samples of *Plasmodium* is to allow accurate relative measures of gametocytes densities and their impact on mosquito infectivity and TRI efficacy (Da et al., 2015). Natural *Pf* samples are supposed to be diluted to generate a wide range of parasite densities and then feed to the mosquitoes.

Similarly, mosquito infection with malaria parasites depends on complex interactions between the mosquito immune response, the parasite developmental program as well as the midgut microbiata. The protozoan *Pf* has a complex life cycle in which asexual multiplication in the vertebrate host alternates with an obligate sexual reproduction in the anopheline mosquito. Apart from the apparent recombination advantages conferred by sex, *Pf* has evolved a remarkable biology and adaptive phenotypes to ensure its transmission despite the dangers of sex. The parasite has the morbid characteristic of being the deadliest protozoan parasite of humans (Habtewold et al., 2015).

2.6 Methods of detecting and quantifying sporozoites

On the best methods of detecting and quantifying sporozoites as a determinant of the malarial infection in the mosquito vector, Bass et al., (2010) wrote that direct observation of the parasite under the microscope is the most reliable method though it requires fresh materials which are often difficult to rear for later stages even if it means in standard laboratories. Moreover, it requires experienced microscopists for accurate procedures. Monoclonal antibodies against the circumsporozoite (CS) protein have been introduced as an alternative to microscope because its sensitivity and specificity is high and parasite quantification is also possible.

Polymerase chain reaction (PCR) has proven to be a more sensitive method to determine malarial infection rate in the mosquitoes as it is able to quantify sporozoites which is the final infective stage of *Pf*. PCR based methods exceeds the sensitivity of microscopic examination with the detection limit between 3-10 sporozoites in a mosquito. Of the currently available sensitive assays for identifying *Plasmodium* from the mosquitoes, each requires salivary gland or midgut dissection, overnight preservation before DNA extraction, southern hybridization, multiple or separate species-specific reactions (nested PCR). Nevertheless, a selection of appropriate primers, storage methods of mosquito samples and foremost extraction methods all can affect PCR performance. Therefore, some proven gold standard methods may give substandard results for detecting *Plasmodium* from mosquitoes. That is why recently, highly specific real-time PCR (RT-PCR) has been developed for this purpose and is showing promising results.

2.7 Factors affecting *Plasmodium* infection into mosquitoes

2.7.1 Transmission efficiency

Although changing hosts is precarious for parasites the probability of successful transfer can be enhanced or reduced by many events. The success rate or efficiency of transmission is a major determinant of, 1) the geographical or host distribution of parasites and 2) the incidence of any given parasite and the disease it brings to its host.

Parasites often enhance the probability of their own transfer. Hosts through their own behavioural patterns unwittingly encourage transmission as well. Thus, parasite transmission usually involves a combination of parasite induced and parasite exploited behaviour on the part of both the vertebrate and invertebrate hosts. The lifecycle of some vector borne parasite seem so complex and inefficient that the biggest unsolved mystery is why these parasites have not become extinct.

Clearly there are elements of transmission efficiency that remain unrecognised or underappreciated. Parasites that employ vectors for their transmission are often the most complex because their success involves adaptation to both vertebrate and invertebrate hosts, the case of *Pf*. Moreover, the parasite success relies on these two very different but equally important hosts (Churcher et al., 2014).

2.7.2 Transmission frequency

The shorter the lifecycle of a parasite the more frequent it must be transferred between hosts and the more efficient its transfer mechanism must be. If parasite causes acute infection that can kill the host, rapid cycling to new non-infected hosts takes an added urgency. It has been argued that vector borne parasites generally cause more severe pathology and mortality in their vertebrate hosts than do non-vector borne parasites. The logic of this interaction lies on the assertion that these parasites cannot afford to become too adapted to their vertebrate hosts and still live successfully within their invertebrate hosts. Of course, some parasites spend their time in each host whilst in a different developmental stage like the case of *Pf* which undergoes sexual development in a mosquito vector so that stage specific adaptations may occur.

Vector borne parasites tend to be transmitted more frequently because the lifespan of the vector is often too short and frequent transmission favours greater pathogenicity. Following this one would expect that parasites with long lived vectors like ticks that also serve as reservoir host might be less pathogenic than mosquitoes that only live for a few days or weeks. Both transmission frequency and efficiency are related to the blood feeding frequency and efficiency of the vector (Akinosoglou et al., 2015)

2.7.3 Vector acquisition of parasites from vertebrates

Ingestion of viable parasites during feeding does not always result in the infection of the vector. One obvious factor that can influence the success of the transfer is the number of parasites (dose) ingested by the vector. If the dose is too small, it may be overcome by the vectors immune system response so that insufficient parasites are available to invoke an infection in the vector. And after ingestion parasites must find their way to targeted organs in the vector where they can develop or replicate.

The path has many curves that may lead to the demise of the parasite therefore understanding the mechanisms and genetics of parasite resistance and susceptibility in the vector is very crucial.

Among other strategies under consideration for interrupting the transmission of the vector borne agents is to engineer vectors that can no longer successfully transmit pathogens and replace wild vector population with these resistant forms (Ndo et al., 2016)

2.7.4 Cyclo-propagative transmission

Protozoan parasites like *Pf* undergo both sexual development and reproduction within the vector. These parasites both increase in number and transform to a different life stage before transmission can take place. The time from when an arthropod ingests an infected blood meal to when it becomes infective to other vertebrate hosts when it again feeds on blood is termed the intrinsic period and can be dependent on the temperatures as it may be longer at cooler temperatures and shorter at higher ambient temperatures.

In contrast the intrinsic incubation period is the time in the vertebrate host from parasite delivery by vector until the vertebrate host is able to infect new host vectors. In most infectious diseases, the intrinsic incubation period is defined as the time from exposure until clinical symptoms appear. More over some malaria parasites are not infectious to vectors until after clinical symptoms have abated (Saunders et al., 2007).

2.7.5 Parasite maintenance

Not all hosts that become infected are essential to perpetuating or maintaining parasites. Because some parasites are transmitted inefficiently they frequently end up in hosts that either cannot support their development or reproduction or those are unlikely to serve as reservoir hosts for the future transmission of parasites. The intake of a plasmodium infected blood meal may affect mosquito physiology and a series of trade-offs may occur in particular between immune defences, reproduction and self-maintenance (Lecona-Valera et al., 2016).

2.7.6 Parasite enhancement of transmission

Carter et al.,(2007) discovered that parasites are not spectators of their own transmission. Many infectious agents participate actively by changing the behaviour or physiology of both their vertebrate and invertebrate hosts. This increases probability of vector transmission.

2.7.7 Vector capacity

It is defined as the dynamic relationship between vectors of infectious disease agents and vertebrate hosts(Gendrin et al., 2016). It combines the physiological attributes of vectors that determine their susceptibility to infection and their ability to transmit pathogens (vector competence) with relevant

ecological and behavioural traits of vectors such as longevity, host preference and abundance. This can be determined by Extrinsic Incubation Period (EIP) which is the time from the uptake of infectious blood meal by the vector to the time the vector is capable of transmitting the pathogens and is mostly influenced by temperature.

2.7.8 Vector competence

Vector competence can be defined as the ability of individuals in a population of arthropods to become infected and to transmit a given strain of a pathogen. Cohuet et al., (2010) argued that not all arthropods that feed upon an infective host become infected. Furthermore, not all that become infected will become infective.

CHAPTER THREE

3.0 RESEARCH DESIGN, MATERIALS AND METHODS

3.1 Type of research study

This was a laboratory experiment in which *Plasmodium falciparum* gametocytes were fed to female *Anopheles gambiae* Kisumu (a susceptible laboratory strain) to investigate malaria transmission.

3.2 Study design

This project required the integration of at least three novel techniques: *in vitro* gametocyte production, membrane feeding, and the molecular or histologic detection of parasites in various stages of development in the mosquito.

The initial study involved the production of purified stage V gametocytes in culture and titration of the number of gametocytes fed to mosquitoes that will result in a detectable infection. Membrane feeding procedures followed in which the sampled female mosquitoes were arranged in equal replicates as per the determined optimal feeding density and fed with blood infected with *Pf* gametocytes artificially. The mosquitoes were starved for at least 12 hours prior to the feeding. The same optimal feeding time was observed for all the feeds.

Parasite load was quantified soon after 24 hours (ookinetes) by analysing gametocytes uptake of the mosquitoes during membrane feeding. From day 4 to 9 the presence of oocysts were checked and estimated through midgut dissections as observed under a light microscope. The oocysts were then observed each day as they grew in size and underwent sporogony to produce a thousand of sporozoites which is the infective stage of the parasite and finally from day 14 to 18 sporozoites were checked and quantified in the mosquito salivary glands by using real-time PCR. In between the time space for checking a particular developmental stage, the infected mosquitoes were incubated and fed with 10% sugar solution. This method allowed for assessment of the various sporogonic stages before, during and after the passage of the mid gut wall.

3.3 Study period

The study was carried out over a four months' period from June to September 2016.

Table 1: Activities and timelines of the study

Activity	Time- frame
Ethical clearance	June 2016
Mosquito rearing and parasite culturing	June-July 2016
Membrane feeding and Observation of *pf* migration in the mosquito mid gut	July 2016
Data management and analysis	July- August 2016
Thesis (final report)	September 2016

3.4 Study site/ place

The study was carried out at Malaria Alert Centre (MAC) of the College of Medicine (COM). The actual experiments were conducted in the insectary facilities at MAC where a colony of *Anopheles gambiae* (Kisumu strain) is kept and the Molecular Lab at COM where parasite culturing procedures were performed.

3.5 Study population and methods

3.5.1 Mosquito rearing

3.5.1.1 Anopheles gambiae

For the purpose of this study, a good number of female *Anopheles* mosquitoes were transferred from the cages and put in separate cages for human blood feeding. The blood feeding was done by placing a human hand in the cage for at least 15 minutes. Determination of fully fed mosquitoes was by observing the down settlement of the fed mosquitoes with full red engorged abdomens. Blood fed mosquitoes were kept in the cage to allow them digest their blood meal and subsequently lay eggs. During these incubation period mosquitoes were provided with 10% sugar solution

through a piece of soaked cotton wool. Under required temperatures and humidity conditions, females took 3-4 days after blood feeding to lay eggs. A simple observation technique for full gravidity of the mosquitoes is turning of the full red engorged abdomen into creamy whitish colour as a result of the presence of the eggs.

Egg collecting cups lined with wet filter papers were placed in the cage to mimic wetenvironmental conditions so that gravid females could lay eggs in them. Eggs were then transferred into rectangular plastic trays filled with mineral water. Larvae hatched after 2 days and were reared on fish food (KOI'S CHOICE) and fed once daily. The pictures below show larvae plastic trays and egg collecting cups.

Figure 3 : Plastic larvae rearing tray filled with mineral water and showing Anopheles larvae

Figure 4: Plastic cups used to collect eggs from gravid Anopheles females and pupae

The full-grown pupae were then collected into plastic cups filled with mineral water and placed in a separate cage where adults emerged after 3-4 days. The emerging adults were fed with 10% sugar solution until they were at least 3 days old when their mouth parts (proboscis) have been fully developed for blood feeding either by direct biting or membrane feeding hence the older the mosquitoes the effective the blood feeding due to the full development of the proboscis to suck the blood efficiently.

3.5.1.2 Collection of Anopheles funestus

3.5.1.2.1 Field collection

Collection of live adult mosquitoes was carried out at Meldrum village in Chikhwawa district in the Lower Shire Valley, approximately 40 km south of Blantyre. Battery operated aspirators of the Prokopack type were used to sample mosquitoes from inside dwelling house. The roofs and walls have been known to be the resting places for mosquitoes after blood feeding on humans. The

collected mosquitoes were then put in a cage and later the blood fed females (shown by blood engorged abdomen) were transferred into paper cups. The numbers per cup depended on the space in the cup to avoid congestion which can result into death as they strive for moisture, space to settle and food within. The paper cups were later put in a cooler box with a wet towel at the bottom (to provide humid conditions) and a sugar soaked cotton wool on the top (for feeding) as they were being transported from the field to the insectary back in Blantyre for rearing.

3.5.1.2.2 Mosquito handling/rearing.

The mosquitoes were allowed to digest the blood meal whilst feeding on 10% sugar solution. After 3 days, they were subjected to forced egg laying where each female mosquito was put in a separate Eppendorf tube (1.5 ml) with a soaked piece of cotton for feeding and a filter paper to provide a favourable environment for egg laying. Under favourable conditions (temperatures of 37 °C and 80-90% humidity) the mosquitoes laid eggs on the filter papers after 3 days and the eggs were transferred in water plastic cups (Fig. 3.2) to allow them hatch into larvae. Larvae hatched after 2 days and were reared on fish food (KOI'S CHOICE). Larvae were then transferred into plastic trays (Fig. 3.1) filled with mineral water to allow them have enough space to mature into pupae. The full-grown pupae were then collected from the plastic trays to egg collecting cups filled with mineral water using pipettes and put in cages where they emerged into adults. As they started to emerge into adults, a piece of cotton wool soaked in 10% sugar solution was put on top of the cage for the emerged adults to feed on waiting for the membrane feeding procedures.

3.5.2 Gametocytes production

Pf parasite culturing was done at the College of Medicine Molecular Laboratory by well trained staff under strict supervision of highly qualified experts. This artificial procedure imitates the natural production of gametocytes.

During parasite culturing, the quality and age of the blood (human blood donated by a healthy person), temperature and serum among other factors are extremely important and can equally affect stress of the culture and ultimately the yield of gametocytes. It is recommended that blood older than 7 days must not be used.

A single stressed flask on day 1 was potentially sub cultured into four or five flasks of developing parasites and the asexual reproduction was monitored throughout the day. Whenever the parasite development slowed or halted due to cell death which hinders early stage development of schizonts,

5-10 ml of fresh medium was added to each stressed culture to supply additional nutrients. All the flasks were shaken gently overnight. The following morning, cultures had at least 10% rings-up to one fifth of these were expected to develop into gametocytes.

For isolation of late stage gametocytes (stage III-V), complete medium was supplemented with 50 mM (final concentration) *N*-acetylglucosamine(GlcNAc) from day 0 onwards and medium replacement was continued for at least 5 days to eliminate the asexual stages. GlcNAc is selectively toxic for trophozoites and schizonts and destroys these stages while not affecting gametocytes development.

For isolation of early stage gametocytes (stage I or II), cultures on day 1 were subjected to 5% sorbital. Unlike very early-stage gametocytes (stage 1), mature asexual parasites (mid trophozoite to schizont) are susceptible to lysis by 5% sorbital and this can be beneficial to rid cultures of asexual parasites. Timing is very crucial as early stage II can be at risk of lysis and early asexual stages (rings) are unaffected by sorbital. After sorbital treatment cultures were returned to daily medium changes in the presence of GlcNAc to inhibit any further asexual growth.

At day 1, cultures containing a mixture of trophozoites and early stage gametocytes were further sub-cultured. Fresh RBC were added and healthy asexual growth was encouraged by expanding the number of flasks with overnight shaking to minimise multiple invasion of individual RBCs. Cultures were processed early on day 2 when they consisted of a mixture of early stage gametocytes and asexual ring stages. Once thoroughly washed free of parasite pigment, gametocytes were magnetically separated from uninfected RBC and ring stage parasites. The process followed all the requirements as stipulated in the standard operating procedures (SOP) of the *in vitro* gametocytes production to yield the required concentrations for the study.

Emphasis was put on maintaining the temperatures at 37 °C to imitate the normal body temperature especially during transportation of the parasites to the mosquitoes.3.5.3 Membrane feeding.

3.5.3.1 Determination of optimal feeding density

To determine the optimal feeding density, varying numbers of mosquitoes were put in 4 different well labelled feeding cups. Mosquitoes in each cup were allowed to feed from uninfected human blood after being starved for not less than 12 hours. Observations were made at specified constant time to estimate the maximum number of mosquitoes per feed. Fully fed mosquitoes were

determined by the mosquito's abdomen being fully engorged with blood (reddish appearance) and their down settlement in the feeding cup. Refer to Appendix 3(i) for the experimental set up to determine the optimal *Anopheles* feeding density.

3.5.3.2 Determination of optimal feeding duration

In order to determine the optimal feeding times, an equal number of mosquitoes was put in 4 different cups and fed with uninfected human blood after being starved for not less than 12 hours. Determination of the number of mosquitoes per cup was deduced from the results of an experiment on optimal feeding density. Observations were made as to when most/all the mosquitoes were fed in each cup. Refer to Appendix 3 (ii) for the experimental design to determine the optimal *Anopheles* feeding duration.

Holding all other factors that might influence feeding of mosquitoes like longer starvation periods, provision of optimal survival temperature in the experimental room as well as feeding the mosquitoes in the dark, the optimal feeding duration for 20 mosquitoes was determined. For validity of results, these experiments were repeated 3 times before determining the optimal *Anopheles* feeding duration.

3.5.3.3 Membrane feeding with cultured Plasmodium falciparum gametocytes

For the purpose of the study, 20 females were transferred from the rearing cage into paper cups for membrane feeding and five replicates were fed giving a total of 100 females being fed per cycle.

Prior to feeding, mosquitoes were starved for at least 12 hours where water and sugar solution were withdrawn. After this starvation period, they were provided with blood infected with P*f* gametocytes for a minimum of 15 minutes. At least 2 ml of the infected blood was pipetted into the glass feeding bell wrapped with parafilm membrane at the bottom to provide a favourable surface for the mosquitoes to bite through. The feeding cup was placed directly below touching the membrane of the glass bell for easy access of the blood by the mosquitoes (Fig. 3).

Figure 5: Anopheles mosquitoes feeding load through membrane feeding apparatus.

Mosquitoes were allowed to feed in the dark so as to mimic the normal conditions in which they feed in the natural environment. Observing for fully fed mosquitoes was determined by the mosquito's abdomen being fully engorged with blood (reddish appearance) and their down settlement in the feeding cup. Refer to Appendix 3 (iii) for the experimental design for feeding *Anopheles* with cultured *Pf* gametocytes.

Due to reasons beyond control (crashing of the parasite line that was being cultured) only *An. gambiae* was membrane fed so the experiments that were conducted were only for this specific species. No experiments were carried out using field collected *An. funestus* population.

The cultured gametocytes were transported in test tubes properly wrapped in tissue paper so that the normal human blood temperature of 37 °C was maintained until they were fed to the starved mosquitoes. Proper handling was emphasized so that the gametocytes were not subjected to thermal

shock. Gametocytes were transferred from the molecular laboratory when the membrane feeding apparatus was already set up and feeding commenced as soon as possible.

All the replicates had the same number of mosquitoes, fixed feeding time and parasite (gametocytes) concentration to avoid bias of results.

It is expected that keeping all conditions favourable (warm temperatures and moisture), blood fed mosquitoes are supposed to fully digest their blood meal after 3-4 days (Vega-Rodríguez et al., 2014).

For detailed list of equipment, materials, laboratory reagents and a step by step procedure for membrane as well as mosquito dissection and mid gut extraction, refer to the full Standard Operating Procedures (SOP) attached (Appendix 1 and 2)

3.5.4 Activities post membrane feeding

Soon after membrane feeding, mosquitoes were stored in a safe locked room (to avoid contamination with the uninfected mosquitoes reared in the insectary), being fed with 10% sugar solution for observation of the sexual developmental stages of the parasite (oocysts and sporozoites).

In between the spaces for each stage checking, the mosquitoes were in incubation so as to allow proper progression of the development stages and they were fed with 10% sugar solution throughout the process for the survival.

3.5.4.1 Gametocytes uptake analysis

In order to determine if the fed mosquitoes have really taken up gametocytes during membrane feeding at least a single mosquito from each cup was taken and analysed for gametocyte uptake by dissecting them in the laboratory and checking for gametocytes presence under a microscope. In total, 10 mosquitoes were used between 24 hours and 48 hours after the membrane feeding.

3.5.4.2 Midgut dissections for oocysts

Presence of oocysts in the midgut indicates that sexual reproduction has taken place in the mosquito vector since this is a stage which develops after male and female gametocytes fertilise to form zygote. At least 2 of the fed mosquitoes were taken from each cup and dissected in the laboratory, have their midgut extracted for observation of the parasite transition from gametocytes to oocysts

under a light microscope. A total of 60 mosquitoes were dissected for oocysts density between day 4 and 9. Refer to SOP for detailed requirements for the procedure (Appendix 2).

Table 2 : A detailed summary schedule of activities that were followed post membrane feeding.

TIME(DAYS)		ACTIVITY	REMARKS (DONE OR NOT)
Day 0		Membrane feeding	Done
Day 1		Remove a single female from each cup for *Pf* PCR	Done
Day 2		Remove a single female from each cup for *Pf* PCR	Done
Day 3		Incubation	Done
Day 4		Remove 2 females from each cup for oocyst dissection	Done
Day 5		Remove 2 females from each cup for oocyst dissection	Done
Day 6		Remove 2 females from each cup for oocyst dissection	Done
Day 7		Remove 2 females from each cup for oocyst dissection	Done
Day 8		Remove 2 females from each cup for oocyst dissection	Done
Day 9		Remove 2 females from each cup for oocyst dissection	Done

3.6.0 Data management and analysis

3.6.1 Data collection tools

Well-designed data collection forms were used (Appendix 3 i, ii, iii, iv). The forms captured all the necessary information like the feeding success of the mosquitoes during the membrane feeds and the infection success of the mosquitoes after determining the gametocytes uptake soon after membrane feeding. The forms for determination of number of oocysts quantities included vital information like replicate number, mosquito number under each replicate, parasite stage at which mosquitoes were fed, incubation period, number of oocysts at each dissection of the mosquito mid guts as observed under the microscope.

Laboratory data collected on predesigned paper based data forms were later entered into a REDCap database and stored on data server at MAC. All paper data forms were properly filed and stored in filing cabinets.

3.6.2 Results presentation

Data collected on parasite densities (oocysts and gametocytes) were summarised into charts and expressed as percentages so was the data for the feeding and infection success of the mosquitoes.

3.7.0 Ethical Considerations

As the study uses humans during mosquito feeding and sampling in the insectary as well as during midgut dissections in the laboratory, participants were fully made aware of the objectives of the study before commencement. Since the people were potentially exposed to mosquito bites and risk of infection during the feeding with infected blood meal, they were made aware of all the possible risks of participation and were given malaria prophylaxis during and one month following the exercise. They were also offered free testing and treatment for malaria with Lumefantrine-Artemether (LA) during the period of the study by a clinician. No personal identifiers were used in data analysis. This ensured confidentiality and safety of all information collected during the study.

Results have been made available only to researchers in the study, securely stored and may be kept for longer periods as may be relevant.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS

4.1 RESULTS

Female *Anopheles* mosquitoes were fed blood meal to maximise their nutritional requirement. To achieve this, a series of experiments were conducted to determine optimal mosquito density in the cups and feeding duration.

4.1.1 Optimal *Anopheles* feeding density

Table 3 shows results of feeding various numbers of *Anopheles* mosquitoes with blood for a period of 15-20 minutes. The highest feeding success (95%; n = 19) was achieved in Cup 4 which had a density of 20 mosquitoes.

The optimal mosquito feeding density using the standard entomological paper cups was concluded to be 20 mosquitoes.

Table 3 : Determination of optimal Anopheles feeding density.

CUP NO.	NO. OF MOSQUITOES/ CUP	DURATION OF FEED (Minutes)	FULLY FED MOSQUITOES	PERCENTAGE OF FED MOSQUITOES
1	50	15-20	19	38.0
2	40	15-20	22	55.0
3	30	15-20	20	66.7
4	20	15-20	19	95.0

4.1.2 Optimal *Anopheles* feeding duration

Table 4 shows results of feeding 20 female *Anopheles* in a standard entomological paper cup for a period of 14, 15, 18 and 20 minutes. All females were fully fed when exposed for more than 15 minutes.

The optimal mosquito feeding duration for a density of 20 mosquitoes in the standard entomological paper cups was concluded to be 15-20 minutes.

Table 4 : Determination of the optimal Anopheles feeding duration (minutes)

CUP NUMBER	NUMBER OF MOSQUITOES	FEEDING DURATION (Minutes)	NUMBER FULLY FED n (%)
1	20	14	19 (95)
2	20	15	20 (100)
3	20	18	20 (100)
4	20	20	20 (100)

4.1.3 *P. falciparum* gametocytes feeding

The Table 5: shows results of feeding infected blood to female Anopheles. All mosquitoes (100%) fed P. falciparum gametocytemic blood.

Replicate #	Anopheles #	Feeding time (min)	Parasite concentration (constant)	Stage of the parasite during feed	Number of fully fed mosquitoes
1	20	15-20	1.32*10^6 gam/ml	Stage V	20
2	20	15-20	1.32*10^6 gam/ml	Stage V	20
3	20	15-20	1.32*10^6 gam/ml	Stage V	20
4	20	15-20	1.32*10^6 gam/ml	Stage V	20
5	20	15-20	1.32*10^6 gam/ml	Stage V	20

4.1.4 Gametocytes uptake analysis

Results of *P. falciparum* gametocytes uptake showed gametocytes observed on midguts of mosquitoes held or incubated at room temperature within 24 hours (100%; n = 5) and no oocysts at 48 hours (0%; n = 5) post membrane feeding. The presence of gametocytes in mosquitoes within 24 hours indicates that gametocytes inoculation was successful.

4.1.5 Mid gut dissections for oocysts

Results of holding mosquitoes longer up to 9 days from 4 days of incubation before dissecting their abdomens are shown in Table 6. Again, no oocyst (0%; n =60) was observed on the mid guts.

Table 6 : A summary of results of holding the mosquitoes longer to 9 days.

Date	Cup Number	Mosquito number	Parasite stage at which mosquitoes were fed	Incubation period	Number of oocysts	Fecundity (Eggs)
21-07-16	1	2	Stage V gametocytes	4 days	0	present
"	2	2	Stage V gametocytes	4 days	0	present
"	3	2	Stage V gametocytes	4 days	0	present
"	4	2	Stage V gametocytes	4 days	0	present
"	5	2	Stage V gametocytes	4 days	0	present
26-07-16	1	2	Stage V gametocytes	9 days	0	present
"	2	2	Stage V gametocytes	9 days	0	present
"	3	2	Stage V gametocytes	9 days	0	present
"	4	2	Stage V gametocytes	9 days	0	present
"	5	2	Stage V gametocytes	9 days	0	present

4.2 DISCUSSION

4.2.1 Optimal *Anopheles* feeding density

The optimal feeding density was concluded to be 20 mosquitoes per the standard entomological paper cups. There was less competition for a blood meal provided through a glass bell at mosquito density of 20 resulting in almost all of them being fed. An increase in the number of mosquitoes per feeding cup will reduce the feeding success as they compete for the blood meal. However, the optimal density might vary according to different cup sizes.

4.2.2 Optimal *Anopheles* feeding duration

The feeding time is an essential factor which influences the membrane feeding success. The feeding success depends on the will of mosquitoes to feed as well as the time of exposure. The longer the time of exposure the higher the chances of the mosquitoes being fully fed since they will have adequate time to reach for the blood meal under the glass bell. The will of the mosquitoes to feed is highly dependent on the starvation time. The longer the starvation time, the increase in the will of the mosquitoes to feed. Mosquitoes were starved for not less than 12 hours prior to blood feeding.

4.2.3 *P. falciparum* gametocytes feeding

The significance of determining feeding density and duration lies primarily in successful malaria parasite (gametocytes) uptake.

The 100% feeding success rate can be attributed to two main experimental factors, 1) optimal mosquito density of 20 females per feeding cup and adequate feeding time of >15 minutes.

Three factors in consideration were: 1) mosquitoes were starved for 12 hours prior to feeding, 2) optimal mosquito densities (20 mosquitoes/ cup) were maintained and they were fed for the optimal duration established during the initial experiments. In additional, the full feeding could have been due to ideal temperature and humidity in the insectary.

4.2.4 Mid gut dissections for oocysts

Pf sexual development in a vector starts as soon as the mosquito ingests an infected blood meal containing asexual forms known as gametocytes. Once inside the vector these gametocytes differentiate into male and female gametes. The male gametes detach themselves from the exflagellation centre and actively start searching for female gametes for fertilisation. The ookinete is the product of that fertilisation. It is this ookinete that transforms into oocyst after passing

through the mid gut epithelial cells to reach the basal lamina. This is the only stage which grows extracellulary and normally starts after day 3 of parasite inoculation up to day 9 when each oocyst grow in size and undergoes sporogony to produce thousands of sporozoites which is the final infective stage of the malaria parasite (Aly et al., 2009).

The 0% result at 48 hours of incubation might indicate a failure of infection despite a successful parasite inoculation in the mosquito vector. Further, it could mean that the incubation period was not adequate enough to allow development of oocysts in the *Anopheles* vector because in nature, wild caught females have been held for 4 – 7 days (Anderson et al 1992 and Mzilahowa et al 2007) before they produce an infection.

While the above reason holds for any incubation less than 5 days, it is not adequate to explain the absence of oocysts beyond 5 days' incubation period.

However, results after 9 days of incubation indicate that there were still no oocysts in the entire extracted mosquito mid guts. This means that parasite development in all the mosquitoes failed even after prolonging the incubation period.

Although changing hosts is precarious for parasites the probability of successful transfer can be enhanced or reduced by many events. Parasites that employ vectors for their transmission are often the most complex because their success involves adaptation to both a vertebrate and invertebrate host, the case of *Plasmodium falciparum*. Moreover, the parasite success relies on these two very different but equally important hosts.

Several factors could have contributed to this infection failure. And these factors are both external and internal of the *Anopheles* host environment.

Specifically, factors that could lead to this failure of possible infection are, but not limited to parasite maturity, transmission efficiency and frequency, vector acquisition of parasites from vertebrates, cyclo-propagative transmission, and parasite maintenance in the vector, parasite enhancement of transmission, vector incrimination, capacity and competence as well as poor handling temperatures as highlighted by Edman et al., (2012) and these have been discussed below.

Mosquitoes might have been fed with immature gametocytes (below stage V) which failed to perpetuate development in the vector.

It is possible that gametocyte culturing did not produce the right age and type of parasites. Gametocytes are supposed to be fed to female *Anopheles* before they exflagellate. It is the drop-in temperature within the invertebrate host that triggers differentiation of male gametes into exflagellation and the subsequent successful mating with the female macrogametes.

Despite the readily available alternative power supply (generator), frequent loss of normal power might have interrupted the *in vitro* parasite culturing process since it failed to imitate the natural development in its vertebrate host. The immature stages have a reduced competence to survive the conditions within the vector and continue their sexual stage development.

Concentration of the parasite (infective dose) differs between different species of the vector. It is possible that the given concentration (1.32*10^6 gam/ml) was not adequate to produce an infection in the vector. (Da et al., 2015) discovered that infection outcome in mosquitoes increase with *Pf* gametocytes density. However, it has to be noted that *Anopheles gambiae* is regarded as one of the best malaria vectors since its long lived, prefers feeding on humans and lives in areas near human habitation as argued by (Cohuet et al., 2010).

Not all vectors which ingest parasites become infected and not all those that are infected become infective as infectivity will not only depend on the dose but also other factors like the suitability of the host to perpetuate and maintain the parasite development or reproduction (Andolina et al., 2015).

The ability of a vector to continue the development of the ingested parasite is called parasite maintenance. It has to be emphasized that not all hosts that become infected are essential to perpetuating or maintaining parasites because some parasites are transmitted in efficiently and they frequently end up in hosts that either cannot support their development or reproduction or that are unlikely to serve as reservoir hosts for the future transmission of parasites.

Gendrin et al.., 2016) defined vector capacity as the dynamic relationship between vectors of infectious disease agents and vertebrate hosts. It combines the physiological attributes of vectors that determine their susceptibility to infection and their ability to transmit pathogens (vector competence) with relevant ecological and behavioural traits of vectors such as longevity, host preference and abundance.

A zero result indicates that the vectors were not susceptible to infection as well as they were not capable of transmitting the pathogens though it has been argued that *An. gambiae s.s* is one of the known and efficient malaria vectors in sub-Saharan Africa including Malawi.

Parasites are not spectators of their own transmission. Many infectious agents participate actively by changing the behaviour or physiology of both their vertebrate and invertebrate hosts. This increases probability of vector transmission.

It has been further argued that vector borne parasites generally cause more severe pathology and mortality in their vertebrate hosts than do non-vector borne parasites. The logic of this lies on the assertion that these parasites cannot show effort to become too adapted to their vertebrate hosts and still live successfully within their invertebrate hosts .Of course some parasites spend their time in each host whilst in a different developmental stage like the case of *Pf* undergoes sexual development in a mosquito vector so stage specific adaptations may occur (Peymanfar et al., 2016).

Internally, ingestion of viable parasites during feeding does not always result in the infection of the vector as after ingestion parasites must find their way to targeted organs in the vector where they can develop or replicate. The road has many curves that may lead to the demise of the parasite therefore understanding the mechanisms and genetics of parasite resistance and susceptibility in the vector is very crucial.

This extends to the immune vector response which might have overcome the parasite immune strength resulting in the weakening of the parasites infectivity. The parasite efficacy might have been reduced.

Parasite efficacy defines the survival of the parasite in its vector. If the conditions within the vector are not favourable for survival the parasite densities are lost and will not be able to progress to another developmental stage since the infective dose has been reduced. If all the parasites are lost then detection will not be possible during analysis (McNamara et al., 2013).

Although protozoan parasites like *Pf* undergo both sexual development and reproduction within the vector, these parasites both increase in number and transform to a different life stage before transmission can take place. The time from when an arthropod ingests an infected blood meal until it becomes infective to other vertebrate hosts when it again feeds on blood is termed the intrinsic

period and can be dependent on the temperatures as it may be longer at cooler temperatures and shorter at higher ambient temperatures (Saunders, 2007).

Externally, handling temperatures are very essential in perpetuating the development of the parasite knowing that malaria parasite thrive at 37 degrees in both the human host and mosquito host immune systems. During the membrane feeds, warm room temperatures and humidity of not less than 80% were observed at all required times through provision of artificial heaters but since the experiments were conducted during cold season (June-July) it is possible that there was a deficiency in maintaining these, but important factors which resulted in the crash of the parasites development in the vector.

Crucially though, handling temperature might also have affected the observation. Malaria parasites (asexual stages) survive at 37 °C in the human host. But sexual stages survive at a lower temperature in the *Anopheles* invertebrate host. The drop-in temperature when transiting between the vertebrate and invertebrate hosts triggers exflagellation of sexual parasite stages.

It was therefore a fundamental requirement that parasites be maintained at 37 °C between the Molecular Laboratory and Insectary. Since there was no specialised equipment to handle parasites at required temperature it is possible that parasites were affected in the process and could not successfully infect the mosquito.

Lastly but not least, the handling temperatures were not stringent enough for the successful maintenance of parasites in their right age and form to result in successful infection of the invertebrate host. Any changes to the gametocytes might result in their destruction within the invertebrate host by the immune system. It is extremely important therefore that moving forward experiments should take into consideration all these factors listed above in order to maximize infection of the *Anopheles* vector.

Others have circumvented the need for temperature maintenance of parasites at 37 °C by using direct feeding on human hosts instead of membrane feeding. However, such experiments have ethical issues to consider.

However, the presence of eggs in all mosquitoes dissected from day 4 confirms successful membrane feeding. This success can be attributed to a series of membrane feeds that were done prior to the one in which mosquitoes were fed with *Pf* laboratory cultured gametocytes. During membrane feeding for determination of optimal feeding density as well as duration, the proper

standards for the procedure were raised, gaps identified and recommendations were made for a successful membrane feeding which were later applied during the main experiment hence this success.

Due to the crash of the parasite line in culture, membrane feeds for field collected *An. funestus* were not done and these results are only for *An. gambiae* therefore are not conclusive for all other vector species since vectors differ in their genetic make-up, morphology, their general behaviours and this makes them differ in the way they adapt to their environment. This makes their role as hosts different as they try to adapt to the changes that might be brought about with the infectious agents. As such, some hosts might be susceptible whilst others are not because the host response as it gets in contact with the parasite is what defines it to be a vector or not.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion.

The fundamental activity of medical entomology is to establish the role that a particular arthropod species or population plays in the transmission of a particular infectious disease.

This study aimed at investigating the sexual development of laboratory cultured *Plasmodium falciparum* gametocytes in *Anopheles* using membrane feeding. The novel approaches of *in vitro* culturing of gametocytes, *Anopheles* rearing and a series of membrane feeds were all designed to achieve this overall objective and were successful.

This study was able to determine the optimal *Anopheles* feeding density and feeding duration. The optimal mosquito feeding density of 20 mosquitoes per feeding cup and the optimal mosquito feeding duration of 15-20 minutes was concluded from the experiment determining the two extremities. With this feeding density mosquitoes are not congested as they strive to feed on the blood meal, moisture as well as the humidity.

Gametocytes inoculation was successful as confirmed by the presence of gametocytes in mosquitoes within 24 hours post membrane feeding. However, the study did not detect sexual stage parasites in mosquito midgut. It was concluded that *P. falciparum* infection into the *Anopheles* was not successful even after holding the mosquitoes for 9 days.

In all, the study was a success because three objectives out of the four were met, membrane feeding was successful and mosquitoes were incubated at the right temperatures and humidity after feeding.

5.2 RECOMMENDATIONS

This study required three novel approaches; *in vitro* parasite culturing of *Plasmodium falciparum* gametocytes, rearing of *Anopheles* vector and membrane feeding. It is therefore recommended that these, being the most critical procedures for the success of the project, they have to be done perfectly and according to the stipulated standards of operating procedures so that the experiments are not interrupted in any way. Maintenance of required handling temperatures at all levels during the implementing of the procedures is very vital to avoid subjecting the cultured parasites to thermal shock which might result to failure of the sexual development of *Pf* in the *Anopheles* vector.

Greater emphasis should be put on maturity of parasites before being membrane fed. Only stage V gametocytes are supposed to be fed to the vectors

Vector acquisition of parasites is highly dependent on the infective dose therefore another study is necessary in which the vectors should be fed with different parasite concentrations so as to determine the optimal parasite concentration that could produce an infection in the Anopheles vector.

Different species of *Anopheles* vector should be membrane fed with cultured gametocytes and observed for sexual development if we are to accurately determine the vector capacity and competence as these results are only for the laboratory reared Kisumu strain and hence can never be conclusive for the whole Anopheles population.

REFERENCES

Akinosoglou, K. A., Bushell, E. S., Ukegbu, C. V., Schlegelmilch, T., Cho, J. S., Redmond, S.,Vlachou, D. (2015). Characterization of Plasmodium developmental transcriptomes in Anopheles gambiae midgut reveals novel regulators of malaria transmission. *Cellular microbiology, 17(2), 254-268.*

Aly, A. S., Vaughan, A. M., & Kappe, S. H. (2009). Malaria parasite development in the mosquito and infection of the mammalian host. *Annual review of microbiology,* 63,(2), 195- 197.

Amoah, L. E., Kakaney, C., Kwansa-Bentum, B., & Kusi, K. A. (2015). Activity of Herbal Medicines on Plasmodium falciparum Gametocytes: Implications for Malaria Transmission in Ghana. *PloS one*, 10(11), 142-168.

Andolina, C., Landier, J., Carrara, V., Chu, C. S., Franetich, J.-F., Roth, A., . . . Snounou, G. (2015). The suitability of laboratory-bred Anopheles cracens for the production of Plasmodium vivax sporozoites. *Malaria journal*, 14(1), 1.

Carter, V., Nacer, A. M., Underhill, A., Sinden, R. E., & Hurd, H. (2007). Minimum requirements for ookinete to oocyst transformation in Plasmodium. *International journal for parasitology*, 37(11), 1221-1232.

Cohuet, A., Harris, C., Robert, V., & Fontenille, D. (2010). Evolutionary forces on Anopheles: what makes a malaria vector? *Trends in parasitology*, 26(3), 130-136.

Da, D. F., Churcher, T. S., Yerbanga, R. S., Yaméogo, B., Sangaré, I., Ouedraogo, J. B., Cohuet, A. (2015). Experimental study of the relationship between Plasmodium gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions. *Experimental parasitology*, 149 (4) 74-83.

Douglas, R. G., Amino, R., Sinnis, P., & Frischknecht, F. (2015). Active migration and passive transport of malaria parasites. *Trends in parasitology*, 31(8), 357-362.

Edmond & Akerman, Sentrfitt E (2012). *Medical Entomology Handbook.* Sieniawka: Palala Press

Gendrin, M., Yerbanga, R. S., Ouedraogo, J. B., Lefèvre, T., Cohuet, A., & Christophides, G. K. (2016). *Differential Effects of Azithromycin, Doxycycline, and Cotrimoxazole in Ingested Blood on the Vectorial Capacity of Malaria Mosquitoes.* Paper presented at the Open Forum Infectious Diseases.Oxford: Oxford University Press.

Ghosh, A., Srinivasan, P., Abraham, E. G., Fujioka, H., & Jacobs-Lorena, M. (2003). Molecular strategies to study Plasmodium–mosquito interactions. *Trends in parasitology, 19*(2), 94-101.

Gouagna, L. C., Mulder, B., Noubissi, E., Tchuinkam, T., & Boudin, C. (1998). The early sporogonic cycle of Plasmodium falciparum in laboratory-infected Anopheles gambiae: an estimation of parasite efficacy. *Tropical Medicine & International Health, 3*(1), 21-28.

Habtewold, T., Groom, Z., Duchateau, L., & Christophides, G. K. (2015). Detection of viable Plasmodium ookinetes in the midguts of Anopheles coluzzi using PMA-qrtPCR. *Parasites & vectors, 8*(1), 1.

Lecona-Valera, A. N., Tao, D., Rodríguez, M. H., López, T., Dinglasan, R. R., & Rodríguez, M. C. (2016). An antibody against an Anopheles albimanus midgut myosin reduces Plasmodium berghei oocyst development. *Parasites & vectors, 9*(1), 1.

McNamara, C. W., Lee, M. C., Lim, C. S., Lim, S. H., Roland, J., Nagle, A., . . . McCormack, S. L. (2013). Targeting Plasmodium PI (4) K to eliminate malaria. *Nature, 504*(7479), 248-253.

Meis, J., Pool, G., Van Gemert, G., Lensen, A., Ponnudurai, T., & Meuwissen, J. T. (1989). Plasmodium falciparum ookinetes migrate intercellularly throughAnopheles stephensi midgut epithelium. *Parasitology research, 76*(1), 13-19.

Ndo, C., Kopya, E., Menze-Djantio, B., Toto, J. C., Awono-Ambene, P., Lycett, G., & Wondji, C. S. (2016). High susceptibility of wild Anopheles funestus to infection with natural Plasmodium falciparum gametocytes using membrane feeding assays. *Parasites & vectors, 9*(1), 1.

Peymanfar, Y., & Taylor-Robinson, A. (2016). Plasmodium Sexual Stage Parasites Present Distinct Targets for Malaria Transmission-Blocking Vaccine Design. *International Journal of Vaccines and Immunization, 2*,(1), 51-56.

Smith, R. C., Vega-Rodríguez, J., & Jacobs-Lorena, M. (2014). The Plasmodium bottleneck: malaria parasite losses in the mosquito vector. *Memórias do Instituto Oswaldo Cruz, 109*(5), 644-661.

Thomas S. Churcher,Robert E. Sinden, Nick J.Edwards, Ian D. Parton, Thomas W. Brock, Jammie T. Griffin, Leannia M. Upton(2014). Probability of transmission of malaria from mosquito to human is regulated by mosquito density in naïve and vaccinated hosts.Downloaded from doi:10.1371.

Vega-Rodríguez, J., Ghosh, A. K., Kanzok, S. M., Dinglasan, R. R., Wang, S., Bongio, N. J., . Pandey, A. (2014). Multiple pathways for Plasmodium ookinete invasion of the mosquito midgut. *Proceedings of the National Academy of Sciences.Downloaded from* https://doi.org/10.1073/pnas.1315517111.

Vlachou, D., Schlegelmilch, T., Runn, E., Mendes, A., & Kafatos, F. C. (2006). The developmental migration of Plasmodium in mosquitoes. *Current opinion in genetics & development, 16*(4), 384-391.

Whitten, M., Shiao, S.-H., & Levashina, E. (2006). Mosquito midguts and malaria: cell biology, compartmentalization and immunology. *Parasite immunology*, 28(4), 121-130.

APPENDICES

1.SOP for Membrane feeding

Malaria Alert Centre Department of Entomology	Copy Number 1	SOP No: 1
Title: Standard Operating Procedure for detection of Plasmodium falciparum parasites in *Anopheles sp* post membrane feed		

	NAME	DATE	Signature
Preparer	Ellen Sithembile Ndhlovu	July 2016	
QA Unit Authority			
Reviewing Authority			
Approval Authority	Dr Themba Mzilahowa		

PURPOSE / APPLICABILITY

Purpose

This protocol establishes procedures of membrane feeding of mosquitoes with human blood artificially infected with plasmodium falciparum gametocytes.

It was developed to ensure this procedure is done correctly for maximum sensitivity and specificity so as to determine malarial infection rate in the female anopheles mosquito vector.

Applicability:

This SOP is applicable to the Entomology Department Manager and personnel, Laboratory Supervisor and personnel and all visiting scientists working within the ICEMR – Molecular Core Laboratories.

ABBREVIATIONS AND TERMS

QA	=	Quality Assurance
QC	=	Quality Control
SOP	=	Standard Operating Procedure
PPE	=	Personal protective equipment
Pf	=	*Plasmodium falciparum*
PCR	=	Polymerase chain reactions

EQUIPMENTS AND MATERIALS

Equipment

- Membrane feeding apparatus
- Water bath
- Temperature controller
- Glass chamber
- Moisture tubes
- Holding valves

Materials

- Mosquitoes
- Aspirators
- *Pf* gametocytes (Stage V)
- PPE (laboratory coats, gloves)
- Feeding cups
- Para film membranes
- Pipettes
- Timer
- Data Form.

Sample

- Human blood

RESPONSIBILITIES

- It is the responsibility of the Supervisor to approve all SOP's
- It is the responsibility of the laboratory personnel working on this protocol to be familiar with this SOP
- Technical staff is responsible for the preparation, review and updating of all SOP's relative to their daily operations.
- QA/QC coordinators are responsible for ensuring that all SOP's are updated annually and meet the standards required.
- Training on SOP's will be conducted upon entry into any position with the ICEMR – Molecular Core.
- It is the responsibility of all the Molecular Core personnel of ICEMR Malawi to be acquainted and up to date with this SOP.

PROCEDURES

Membrane feeding

- Sample female anopheles mosquitoes from the cages
- Arrange feeding cups to be used during the process
- Using aspirators transfer the mosquitoes to the feeding cups according to the required design of the study
- Starve the mosquitoes for not less than 3 hours
- Set up the membrane feeding apparatus (regulating the temperatures of the water bath to 37 degrees Celsius, imitating the normal body temperature.
- Pipette at least 2mls of infected blood into the feeding glass jar wrapped with Para film membrane at the bottom
- Place the feeding cup directly below touching the membrane on the glass jar

- Feed the mosquitoes for at least 15 minutes
- Observe for fully fed mosquitoes
- Repeat the procedure for all the feeding cups until all the mosquitoes in the study are fed
- Keep the cups in a separate locked room at the insectary for observation developmental stages of *Pf* parasites in the mosquito vector

N.B: PPE's SHOULD BE WORN THROUGHOUT THE PROCESS

Female mosquitoes are sampled from the cages and put in feeding cups as per the study design. Preferably, the maximum should be 20 mosquitoes per cup. These mosquitoes are starved for not less than 3 hours before being fed with blood artificially infected with *Pf* parasite gametocytes (cultured at COM laboratory) for at least 15 minutes. At least 2mls of the infected blood is pipetted into the glass feeding jar wrapped with parafilm membrane at the bottom to provide a favourable surface for the proboscis of the mosquitoes during feeding. The feeding cup is then placed directly touching the membrane of the glass container for easy access of the blood by the mosquitoes. Observing for fully fed mosquitoes can be determined by their abdomen being fully engrossed with blood and their down settlement in the cup. For observation of the sexual developmental stages of the parasite, a defined number of the fed mosquitoes are taken from each cup and dissected in the laboratory, have their mid gut extracted for observation of the parasite transition at each stage.

ATTACHMENTS

- No attachments for this SOP

2. SOP for mid gut dissection

Malaria Alert Centre Department of Entomology	Copy Number 1	SOP No: 1

Title: Standard Operating Procedure for detection of Plasmodium falciparum parasites in *Anopheles sp* post membrane feed			
	NAME	DATE	Signature
Preparer	Ellen Sithembile Ndhlovu	July 2016	
QA Unit Authority			
Reviewing Authority			
Approval Authority	Dr Themba Mzilahowa		

PURPOSE / APPLICABILITY

Purpose

This protocol establishes procedures involved in mid gut/ salivary glands dissection to determine oocysts density as well as quantify sporozoites

It was developed to ensure this procedure is done correctly for maximum sensitivity and specificity so as to determine malarial infection rate in the female anopheles mosquito vector

Applicability

This SOP is applicable to the Entomology Department Manager and personnel, Laboratory Supervisor and personnel and all visiting scientist working within the ICEMR – Molecular Core Laboratories.

ABBREVIATIONS AND TERMS

QA	=	Quality Assurance
QC	=	Quality Control

SOP	=	Standard Operating Procedure
PPE	=	Personal protective equipment
Pf	=	*Plasmodium falciparum*
PCR	=	Polymerase chain reactions

EQUIPMENTS AND MATERIALS

Equipment

- Microscope
- Slides
- Dissecting pins
- Forceps

Materials

- Mosquitoes
- Aspirators
- PPE (laboratory coats, gloves)
- Feeding cups
- Pipette
- Data Forms

Reagents

- Normal saline

RESPONSIBILITIES

- It is the responsibility of the Supervisor to approve all SOP's
- It is the responsibility of the laboratory personnel working on this protocol to be familiar with this SOP

- Technical staff is responsible for the preparation, review and updating of all SOP's relative to their daily operations.
- QA/QC coordinators are responsible for ensuring that all SOP's are updated annually and meet the standards required.
- Training on SOP's will be conducted upon entry into any position with the ICEMR – Molecular Core.
- It is the responsibility of all the Molecular Core personnel of ICEMR Malawi to be acquainted and up to date with this SOP.

PROCEDURES

- Mosquito abdomen dissection and mid gut extraction
- Select a few mosquitoes from each of the fed cups to be checked for oocysts or sporozoites
- Set the microscope
- Freeze the mosquitoes at 20 degrees Celsius for at least 5 minutes. This acts as anaesthesia to the mosquitoes.
- Using a forceps/dissecting pin place a mosquito on a slide
- Put some few drops of normal saline besides the mosquito
- Hold the mosquito on the thorax using forceps while using the other

  Forceps/dissecting pin pull the abdomen from the third segment down

  until the midgut is exposed.

- Spread the exposed contents of the abdomen on the water to clearly

  extract the mid gut

- Observe for oocysts on the mid gut
- qPCR analysis of mid guts and salivary glands
- Stain the mid guts and salivary glands
- Determine/quantify oocysts/sporozoites infection/density

N.B PPE's SHOULD BE WORN THROUGHOUT THE PROCESS.

ATTACHMENTS

- No attachments for this SOP

3.Data collection forms

(i) The experimental set up to determine the optimal *Anopheles* feeding density

CUP NO.	NO. OF MOSQUITOES	DURATION OF FEED	NO. OF FED MOSQUITOES	PERCENTAGE OF FED MOSQUITOES
A	50	15-20 minutes		
B	40	15-20 minutes		
C	30	15-20 minutes		
D	20	15-20 minutes		

(**ii) The experimental design to determine of the optimal *Anopheles* feeding duration**

CUP NO.	NO. OF MOSQUITOES	NO. OF FULLY FED MOSQUITOES	PERCENTAGE OF FULLY FED	OBSERVATION FEEDING TIME
A	20			
B	20			
C	20			
D	20			

(iii) Experimental design for *Anopheles* fed with the cultured *Pf* gametocytes

Replicate #	*Anopheles* #	Feeding time (min)	Parasite concentration(constant)	Stage of the parasite during feed
1	20	15-20	1.32*10^6 gam/ml	Stage V
2	20	15-20	1.32*10^6 gam/ml	Stage V
3	20	15-20	1.32*10^6 gam/ml	Stage V
4	20	15-20	1.32*10^6 gam/ml	Stage V
5	20	15-20	1.32*10^6 gam/ml	Stage V

(iv) Form for oocyst detection

Date	Feeding cup Number	Mosquito number	Parasite stage at which mosquitoes were fed	Incubation period	Number of oocysts
