OPTIMIZING AND EVALUATING A NOVEL HIGH THROUGHPUT LAMP ASSAY FOR DETECTION OF LOW INTENSITY *PLASMODIUM FALCIPARUM* INFECTIONS

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Optimizing and Evaluating a Novel High Throughput Lamp Assay for Detection of Low Intensity *Plasmodium Falciparum* Infections

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Laboratory Science of the Jomo Kenyatta University of Agriculture And Technology

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DECLARATION

This thesis is my original work and has not been presented for a degree i university.in any other university

Signature …….Date……

Kenny Kimani Kamau

This thesis has been submitted for examination with our approval as university supervisors.

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DEDICATION

This work is dedicated to my parents Mr and Mrs Kamau, my lovely wife Jackline Kimani, my children Mila Pendo Karimi and Mei Zuri Mweni for giving me an easy time during my studies.

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I would like to acknowledge God for the provision of good health during my research work. My supervisors Dr. Amos Mbugua and Francis Kimani for their profound support and guidance. Kemri staff for always being there when I needed guidance and JICA-PAUSTI for partially funding this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

ACTs	Artemisinin-based Combination Therapy
B3	Backward inner primer
BIP	Backward inner primer
DBS	Dry blood spots
DMLSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
F3	Forward outer primer
FIP	Forward inner primer
HT LAMP	High throughput Loop mediated isothermal amplification
HTL	High Throughput Loop Mediated Isothermal Amplification
IRNS	Intermittent Residual Spraying
LBP	Loop Backward primer
LFP	Loop forward primer
LLINS	Long Lasting Insect Treated Mosquito Nets
MOH	Ministry of Health
NPV	Negative predictive value
PPV	Positive predictive value

RDT Rapid diagnostic test

WHO World Health Organization

ABSTRACT

The prevalence of malaria has been on the decline globally and optimism for the effective control of malaria has been growing. Many countries are making the shift from simply bringing down the number of new malaria cases to aiming for the elimination of malaria altogether. Supporting this bold ambition is the World Health Organization (WHO) which has guidelines for both how this goal may be attained as well as for how countries can obtain WHO certification of the elimination of malaria. Achieving the elimination milestone requires identification and treatment of all parasite carriers, both symptomatic and asymptomatic. There is therefore need to develop improved high throughput diagnostics, with capacity to detect low parasitaemia malaria infections. This would enable early and comprehensive identification and treatment of all asymptomatic cases especially in traditionally non-endemic areas that play a major role in malaria transmission. The aim of this project was to optimize and evaluate a Novel high–throughput loop mediated isothemal amplification (HT LAMP) assay for detection of low intensity *P. falciparum* infections. The assay was modified to enhance throughput and optimized by inclusion of DMSO to increase sensitivity and specificity. An experimental study design was used to evaluate 134 dried blood spot (DBS) samples from children in Busia county, a malaria endemic area in Western Kenya. Nested PCR was used as the gold standard method for detection of low parasitaemia infections that are undetectable by conventional light microscopy. The results indicated that HT-LAMP has a sensitivity of 93.5%, specificity of 100 %, a positive predictive value of 100% and a negative predictive value of 96.7%. HT-LAMP exhibited a strong agreement with nested PCR (k= 0.950). HT LAMP therefore showed superior diagnostic capacity just like that of Nested PCR and thus it can be used in field surveillance to complement malaria elimination interventions. Further optimizations should also be explored to make a multiplex HT-LAMP assay that can detect other *Plasmodium* species.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Over the past ten years, substantial changes have occurred in the prevalence of malaria. Global malaria cases recorded were 228 million in 2018 compared to 231 million cases in 2017(World Health Organization, 2019) The World Health Organization (WHO) reported that 93% of the cases in this time period were recorded in Africa compared to 3.4% and 2.1% from South-East Asia and the Eastern Mediterranean region respectively (World Health Organization 2019). The malaria incidence rate is estimated to have declined globally between 2010 and 2018, from 71 to 57 cases per 1000 population at risk (World Health Organization, 2019).

Additionally, In 2018, there were an estimated 405 000 deaths from malaria globally, compared with 416 000 estimated deaths in 2017, and 585 000 in 2010 (World Health Organization, 2019). The most vulnerable group affected by malaria was Children aged under 5 years. It was documented that they accounted for 67% (272 000) of all malaria deaths worldwide while 94% of all malaria deaths in 2018 were recorded in the African Region (World Health Organization, 2019).

This overall decline in global malaria prevalence has been attributed to concerted global efforts in applying interventions to interrupt *Plasmodium* parasite transmission (World Health Organization, 2015). These include: vector control using long-lasting insect treated mosquito nets, intermittent residual spraying and enhanced case management through improved detection and treatment of cases using artemisinin-based combination therapy (World Health Organization, 2015).

However, effective malaria control continues to be threatened by the rise and spread of drug and insecticide resistant parasites and mosquito species respectively (Sougoufara et al., 2017). Since 2010, about 60 countries have reported incidences of resistance to at

least one insecticide class used in IRS or ITN. Drug resistance has predominantly been observed towards pyrethroids (World Health Organization, 2017).

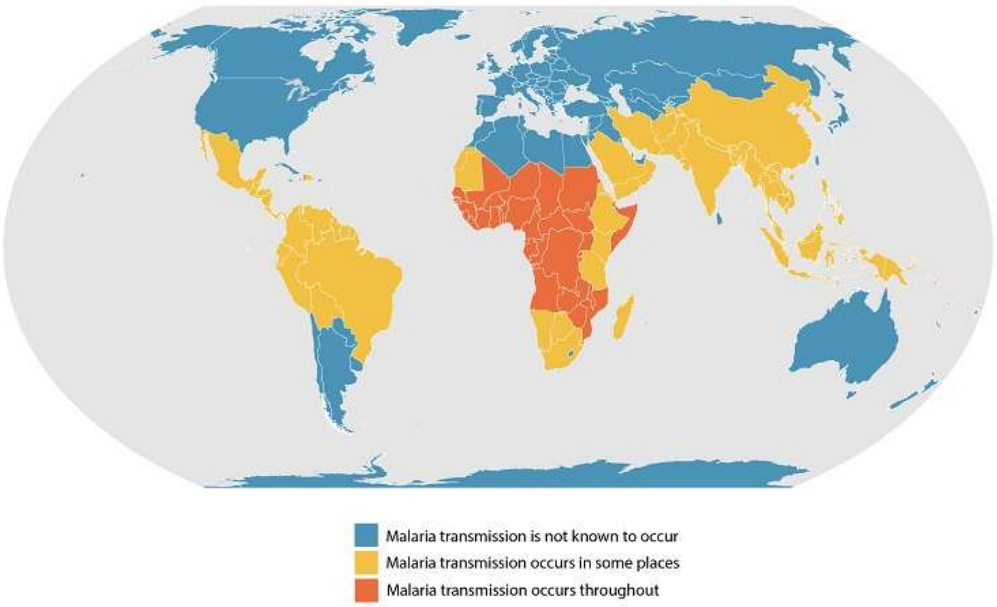
The diagnosis of malaria which has traditionally relied on microscopy has witnessed recent advancements including the deployment of rapid diagnostic tests (RDTs). All the currently marketed RDTs specifically detect one or more of the following *Plasmodium* parasite antigens: histidine-rich protein 2 (HRP-2), lactate dehydrogenase (pLDH), and aldolase (Lucchi et al., 2013). Those that target aldolase and lactate dehydrogenase enzymes are genus-specific and are used to detect all the four major *Plasmodium* species: *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae* (Kudyba et al., 2019). Rapid diagnostic test that target HRP-2 gene are only specific to *P. falciparum* since histidine-rich protein –2 is only present in this species (Lucchi et al., 2013) . However, some *Plasmodium falciparum* parasites in parts of South America and Africa have been recently identified where the hrp-2 gene has been deleted. This may compromise the accuracy of the HRP-2 based RDTs due to potential false negatives (Lucchi et al., 2013)

Despite being the gold standard for clinical diagnosis of malaria, expert microscopy requires rigorous and continuous training in order to develop highly trained microscopists (World Health Organization 2017). Molecular tools have demonstrated greater sensitivity in detecting low parasite infections and correctly identifying the species of malaria parasite (Fernandes et al., 2016). These tools range from conventional PCR-based assays and real-time PCR assays to isothermal amplification assays (Britton et al., 2016). When choosing the type of molecular assay to use, it is paramount to consider cost, robustness and ease of use. Loop-mediated isothermal amplification (LAMP) assays display many of these characteristics and hold promise form field application in malaria diagnosis and surveillance (McCreesh P. 2015).

LAMP uses a thermal stable enzyme BST DNA polymerase that does not need repeated temperature changes typical of PCR. This characteristic makes this technique amenable for adoption to use in field surveillance of malaria cases (McCreesh P. 2015). LAMP

end product amplicon detection can be done visually by checking for the establishment of magnesium pyrophosphate precipitate (Oriero et al., 2015). End point detection of LAMP PCR products has previously been achieved by use of Metal ion indicators, such as calcein, hydroxynaphthol blue and pico-green. in real time detection, melt curve analysis (a bioluminescent output in real time (BART) and lateral flow dipstick and a moveable fluorescence detection unit have also been used (Oriero et al., 2015).

As interventions to control and eliminate the cases of malaria continue to be deployed, low parasitaemia asymptomatic infections will pose a challenge towards achievement of the elimination goal. Molecular tools that can accurately and efficiently detect such low intensity infections would be a welcome addition to existing malaria control toolkit. The aim of this study was to optimize a high throughput LAMP (HT LAMP) assay for the detection of low intensity *Plasmodium falciparum* infections from dried blood spots (DBS).



Source: (www.cdc.gov/malaria/about/distribution.html)

Figure 1.1: Malaria Map around the World

1.2 Problem Statement

Currently within the global sphere, malaria detection and mitigation methods have been limited to two diagnosis detection methods namely; rapid diagnostic tests (RDTs) and Microscopy (Lalloo et al., 2016). Microscopy tests are based on smeared blood stains which are cumbersome, not controllable and have a detection limit of 50 asexual parasites/μL of blood, while an expert microscopist will struggle to detect infections <20 parasites/μL regularly. Microscopy is difficult since it requires highly trained microscopists (WHO, 2014). In addition, a two to three level variance exists between quantification of organisms and microscopists (Lucchi et al., 2013).

Rapid diagnostic tests have been designed to detect the genus-specific aldolase, lactate dehydrogenase enzymes, and *Plasmodium falciparum* histidine-rich protein –2 gene. They are not able to detect low density parasite infection, quantitate or differentiate

between the species. (Lucchi et al., 2013) questioning utilization of the HRP-2 based RDT tests resulting to erroneous negatives in clinical and asymptomatic cases.

Often patients with low infections lack clinical symptoms and may not present to clinics for testing, hence the need for more sensitive, specific low cost and high throughput capacity methods to be developed for deployment in field evaluations. Molecular techniques have shown higher sensitivity and specificity in diagnosis of malaria, their demerit being expensive and impossible to deploy in surveys. The figure below shows the diagnostic detection limit of diagnostic methods used in Malaria diagnosis. Molecular methods i.e. lamp assay for low parasitemia may thus be more ideal for malaria survey of populations.

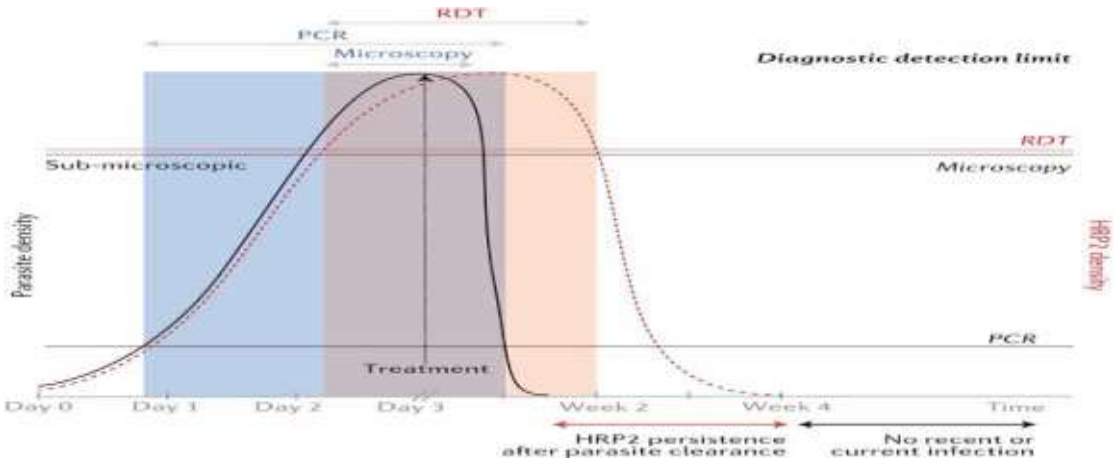


Figure 1.2: Comparison of Malaria diagnostic methods (Wu et. al. 2015)

1.3 Justification of the study

Convectional PCR is sensitive but expensive than LAMP. Loop mediated isothermal amplification is a new molecular amplification technique that is relatively low cost and may be easier to use than other molecular techniques. It uses a water bath at a constant temperature unlike PCR where you use a thermocycler. Its application in epidemiological surveys will help in detection of asymptomatic cases that act as reservoirs of malaria (Hartmeyer et al., 2019).

This will help in achieving stainable development goal (SDG) number 3 to Ensure healthy lives and promote well-being for all at all ages. Moreover, it will help the World Health Organization achieve its Global Technical Strategy for Malaria plan for 2016-2030(World Health Organization, 2019).

1.4 Hypothesis

1.4.1 Null Hypothesis

The novel high throughput (HT) LAMP assay cannot detect *Plasmodium Falciparum* parasites compared to Nested PCR

1.5 Objectives

1.5.1 Broad Objectives

To optimize and evaluate a novel high –throughput (HT) LAMP assay for the detection of low intensity *Plasmodium falciparum* infections

1.5.2 Specific Objectives

1. To optimize a novel high throughput LAMP assay for the detection of low intensity *Plasmodium falciparum* infections from archived DBS.
2. To evaluate the high throughput (HT) LAMP assay against archived malaria samples**.**

CHAPTER TWO

LITERATURE REVIEW

Malaria is a parasitic disease which is potentially fatal communicable to human beings via infected female Anopheles mosquitoes bites (Milner, 2018).

2.1 Epidemiology of malaria

Despite malaria intervention policies and strategies, an estimated 228 million cases of malaria occurred worldwide in 2018, with Africa accounting for about 93% of the global malaria incidences (World Health Organization, 2019). Moreover, global mortality rate has been estimated to be 0.4 million deaths with WHO African Region accounting for 94% of all malaria deaths in 2018 (World Health Organization, 2019). As thus, 0.27 million children have been documented to have succumbed to malaria in 2018. (World Health Organization, 2019). In Kenya, nearly 16.0 million malaria incidences and 40,000 deaths were reported in the year 2013. Although about 55% of mortality and morbidity attributed to malaria is caused by *P. falciparum*,(Snow et al., 2005) *Plasmodium vivax* also causes a significant burden of disease(Guerra et al., 2010).

2.2 Biology of malaria parasite

Seven strains of the parasite are responsible for malaria development in human beings of the genus Plasmodium i.e. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium cynomolgi*, *Plasmodium Simium*, *Plasmodium malariae*, *Plasmodium knowlesi* and *Plasmodium ovale* (Grigg & Snounou, 2017).

There exists a total of 400 various groups of Anopheles mosquitoes, however out of this group only 30 are known to be of serious consequences (WHO, 2014). Recently, malaria cases in human as a result of *Plasmodium knowlesi* have been discovered – having been caused by certain monkey species in South East-Asia forests (World Health Organization, 2015).

However, *Plasmodium falciparum* with *Plasmodium vivax* malaria constitute the highest health cases (World Health Organization, 2019). *Plasmodium falciparum* has been highly prevailing it is most prevalent within the African countries, responsible for majority of malaria mortality(WHO 2017). *Plasmodium vivax* covers a larger region than *Plasmodium falciparum* due to its ability to exist within Anopheles mosquito vector in areas with minimal temperatures, but can also exist in high altitude as well as cool climatic conditions(WHO 2017).

Additionally, it has been characterized by a stagnating liver stage namely, hypnozoite which becomes evident after several months after the first infection that results to recurring medical conditions (WHO 2017).Stagnant stages aid in the survival of *P. vivax* for longer durations with the absence of Anopheles for example in the winter season. Even though *P. vivax* exists in Africa, chances of being infected by the organism are minimal due to lack of the Duffy species responsible for producing protein that necessitates for *P. vivax* infestation in the red blood cells(World Health Organization, 2015) Beyond the African region, *P. vivax* infections are prevalent compared to infections by P. falciparum, resulting in high mortality rates (World Health Organization, 2015).

2.3 Malaria Life Cycle

Malaria series of stages is based on two organisms namely; a vertebrate host (human) and the insect vector (Anopheles mosquito).

2.3.1 Human Lifecycle

When sucking blood from human the malaria infected Anopheles mosquito unloads within the human organism sporozoites, it then follows the transportation of Sporozoits in the blood stream within the liver infecting the liver cells resulting into the development of schizonts (PMI, 2016).

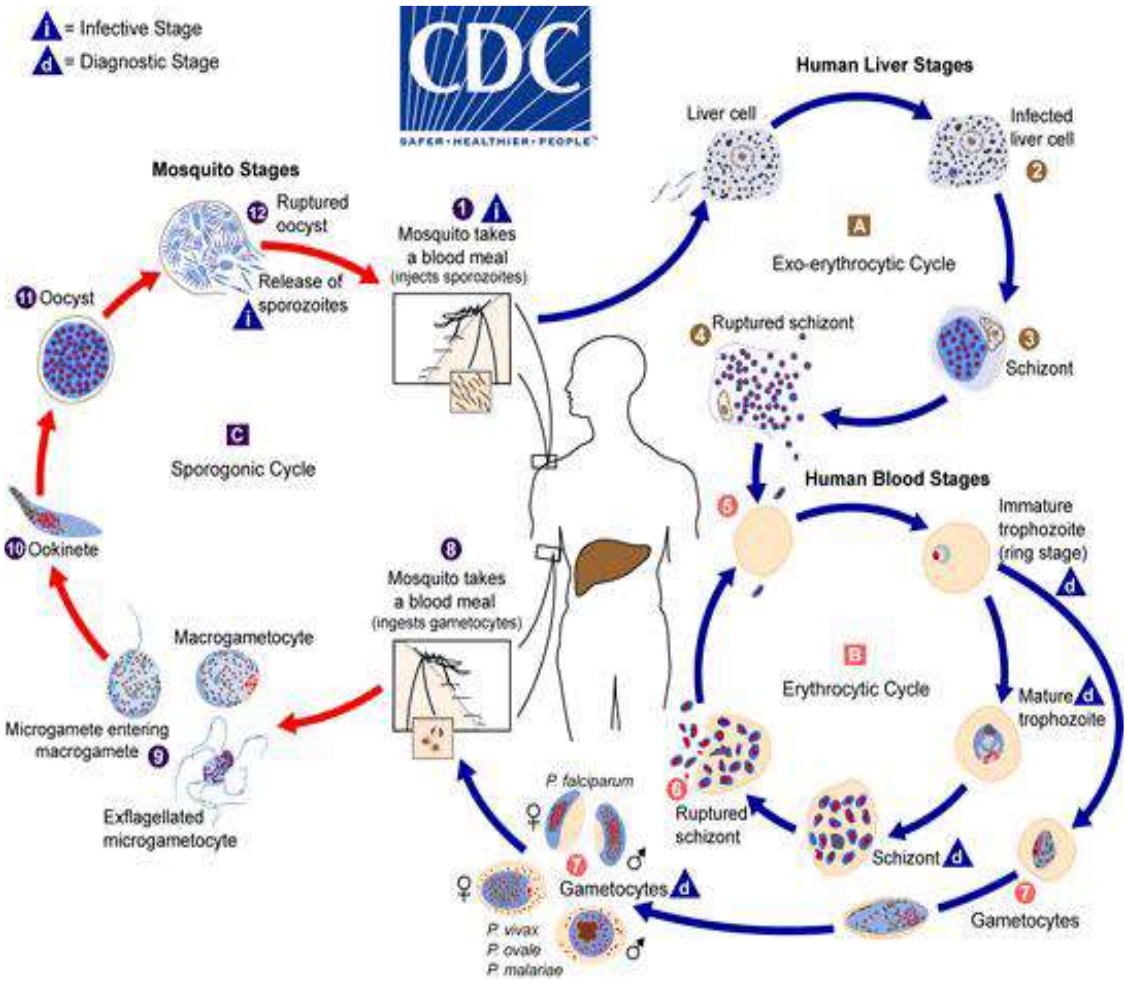
The schizonts rapture releasing merozoites this phase is known as exo –erythrocytic schizogony–,in *plasmodium Ovale* and *P. vivax* a stagnant stage or (hypnozoites) with a prolonged existence in the liver results into further deterioration through infection within the bloodstream for an extended period of time(PMI, 2016).

Exo–erythrocytic schizogony culminates into production of merozoites which then results into the infection of red blood cells and results to sexual reproduction in within the erythrocytes also known as erythrocytic schizogony(Day & Africa, 2017).

Then follows the ring stage trophozoites which develop into schizonts, rapturing and releasing merozoites. Other parasites' develop into sexual erythrocytic levels; microgametocytes (male gametocytes) and macro gametocytes (female gametocytes)(WHO, 2014).

2.3.2 Vector Life Cycle

Vector life cycle is referred to as sporogonic cycle; a female Anopheles mosquito ingests the gametocytes during a blood meal. In the gut of the mosquito the microgamete penetrates the macrogamete resulting to zygotes (sexual reproduction). The zygotes then turn into ookinites which are elongated and motile and they move to the midgut wall of the mosquito where they mature into oocysts, they multiply and breach to discharge sporozoites. The sporozoites move to the salivary glands and are injected into a fresh human host during the next meal. This takes 10 -18 days(PMI, 2016)



Source: (https://www.cdc.gov/malaria/about/biology/index.html)

Figure 2.1: Malaria life cycle. Malaria Symptoms

After a mosquito bite in non-immune persons the symptoms usually appear 10–15 days, patients present with fever, headache, and chills. This then progresses to illness within 24 hours, and may lead to death if not treated. Moreover, children often present with extremely low hemoglobin levels, difficulty in breathing in relation to metabolic acidosis (PMI, 2016).

2.4 Malaria Diagnosis

Previously there have been suggestions that Malaria diagnosis relied on two diagnostic tools; RDT and Microscopy, however gradually molecular diagnosis has been embraced and is currently being used in diagnosis in the goal towards malaria elimination. The various diagnostic methods are discussed as below:

2.4.1 Microscopy diagnosis of Malaria

Microscopic diagnosis entails using blood smears to check for malaria parasites and differentiating between the species. Thick and thin smears are prepared and stained smears are stained for observation. This has been used as the gold standard for any new detection tool However, it still has some precincts i.e.; being laborious, relies on a well-trained microscopists (Kasetsiriku et al., 2016)

2.4.2 Rapid Diagnostic Test of Malaria

Rapid diagnostic tests use immunochromatographic detection of circulating parasite antigens or detect the genus-specific aldolase and lactate dehydrogenase enzymes. Moreover, majority of the RDTs are specific for the P. falciparum histidine-rich protein –2 (Pf HRP-2). RDTs have supplemented some of the bottle necks found in microscopy since it's easy to use and to train on its use, no 'weariness' More ever RDTs are stress-free to supply hence are preferred for point of care (Kasetsirikul et al., 2016).

2.4.3 Molecular Techniques used in diagnosis of Malaria

Molecular tests have been developed in the quest to improve diagnosis of malaria in that they have improved sensitivity and specificity as a result, imprompt treatment has been achieved.(Shahwani, *et,al*. 2016)

2.4.3.1 Polymerase Chain Reaction

PCR uses heat to separate double stranded DNA template a process known as denaturation, what follows is lowering of the temperature to about 72^{o} C this allows attachment of primers to their complementary sequences on the template through a process known as annealing. The primers target 18s RNA gene. This allows the DNA template to add nucleotides with the help of DNA polymerase. This results to formation of double-stranded products this techniques has significantly improved and has helped in studies of genes resistance (Tavares et al., 2011)

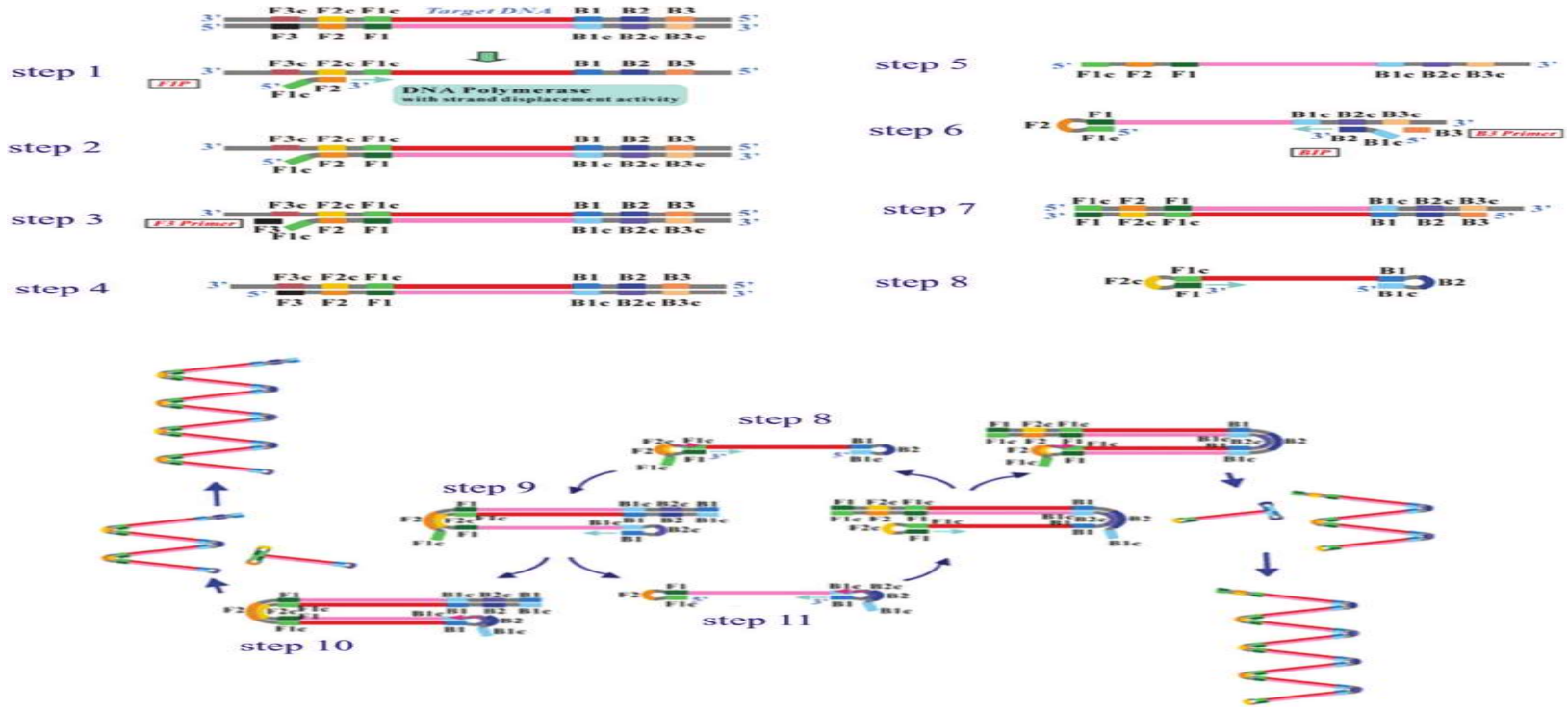
2.4.3.2 Real-Time Polymerase Chain Reaction (RT-PCR)

Real time PCR is based on PCR technique however the difference being that the PCR process can be tracked in real time. Performance characteristics of the mother technique are maintained and combined with primers, probes or dyes that bind with the region of interest e.g. K13 mutation gene (Tavares et al., 2011). The Fluorescent signals are produced as the cycles go on and are collected by a digital camera and a fluorometer. This technique has helped in estimating the quantity of parasites and assessing the extent of infection. (Tavares et al., 2011)

2.4.3.3 Loop-Mediated Isothermal Amplification (LAMP)

This is a technique that amplifies genetic material under isothermal conditions; it has great performance characteristics in terms of sensitivity and specificity. It uses DNA polymerase and four primers that are made to identify six different disarrays of the gene of target (Hayashida et al., 2017) LAMP primers include Forward inner primers (FIP), Backward inner primers (BP), Forward outer primers (F3), Backward outer primers and loop primers ; loop forward primer and loop backward primers that increases the speed of the reaction. (Notomi et al., 2000)The method requires isothermal conditions of about 65^{o}c. LAMP primers anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement

activity, displacing and releasing a single stranded DNA. (Notomi et al., 2000)With the LAMP method, there is no need for heat denaturation of the double stranded DNA into a single strand. The following amplification mechanism explains from when the FIP anneals to such released single stranded template DNA



(Adapted from Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan)

Figure 2.2: Diagram representing the mechanism of the loop mediated isothermal amplification, A) LAMP initial step and B) elongation and recycling step

The forward inner primer (FIP), which contains the sequence from the sense and antisense strands of the target DNA, synthesizes the first strand and thereby initiates the LAMP process (steps 1 and 2). The outer forward primer (F3) hybridizes and replaces the first synthesized strand containing the complementary strand bound to the inner primer, resulting in a stem-loop DNA structure at one end (steps 3-6). This strand initiates the process of forming a dumbbell shape by allowing the inner reverse primer (BIP) to hybridize to the other end of the target DNA, causing displacement of the strand by the outer reverse primer (B3) (BIP). Step 6 to 8). It is then rapidly converted to stem-loop DNA by self-primed DNA synthesis. FIP anneals to the single-stranded region of stem-loop DNA and primer-strand-substituted DNA synthesis, releasing previously synthesized strands. This released single strand forms a stem-loop structure at the 3'end. It then begins DNA synthesis at the 3'end of the B1 region and uses itself as a template to release the complementary strand linked to the FIP (step 9). The released single strand then forms a dumbbell-like structure containing complementary F1, F1c and B1c and B1 regions, respectively (step 11). This structure is the turnover structure formed in step 8. Similar to steps 8 through 11, the structure of step 11 results in self-priming DNA synthesis starting from the 3'end of the B1 region. In addition, BIP attaches to the B2c region and performs primed strand substitution DNA synthesis to release the B1 primed DNA strand. Similarly, the structure is generated in steps 9 and 10, and the same structure is generated in step 8. For the structure prepared in step 10, the BIP will anneal to the single-stranded B2c region, DNA synthesis will continue, and the double-stranded DNA sequence will be replaced. The result of this process is the formation of differently sized amplicon, consisting of alternating reverse iterations of target sequences on the same strand. The product is a different sized structure composed of alternating reverse iterations of the target sequence on the same strand, resulting in a cauliflower-like DNA structure. (Notomi, et al., 2000).

2.5 Treatment of Malaria

2.5.1 ACT and low-dose primaquine

Positive diagnosis of malaria ought to be treated promptly using the WHO recommended ACT and primaquine administered in low dose. ACT mode of action entails killing the asexual stage P. falciparum parasites this is achieved by preventing gametocytogenesis.(Landier et al., 2016) Artemisinins act against the stages I–IV gametocytes, however, it's not able to kill mature infectious gametocytes (stage V) Primaquine has demonstrated the ability to work against mature *P. falciparum* gametocytes, additionally its advised that it's given early together with an ACT for effective results (Landier et al., 2016)

2.5.2 Chemoprevention in pregnant women and children

The WHO recommends administration of at least three jabs of sulfadoxine-pyrimethamine to pregnant women and children. The8se doses are administered to women during the antenatal care (ANC) clinics and in conjunction with the normal vaccinations in children. They are meant to protect the mother throughout the pregnancy period.(WHO, 2017) This has been implemented in the Sub Saharan Africa and has been proved to work in prevention of Malaria and consequently reducing maternal deaths.(Munyekenye et al., 2005)

2.5.3 Vector Control

WHO has recommended the control of mosquito as the most effective way of controlling the spread of malaria, this refers to the use of methods that limit or eradicate the agents which transmit disease pathogens. It entails the following methods: (World Health Organization, 2015)

2.5.3.1 Long Lasting Insecticides Treated Mosquito Nets

Long lasting insecticide treated mosquito nets are recommended to be used in populations susceptible to getting malaria. (WHO 2017)The World health organization proposes that the nets have a longevity period of 3 years, to achieve this public health programs came up with a programme of giving free nets to ensure equal access during the anti-natal visits. In parallel, WHO recommends communication strategies adopted to ensure proper use and maintenance of the nets (WHO, 2017)

2.5.3.3 Indoor Residual Spraying

WHO recommends that to achieve full potential of indoor residual spraying, at most all houses in the regions purposed to be at risk should be sprayed, its effective for 3–6 months. (Lalloo et al., 2016)Longevity of the insecticide is based on the making and the surface sprayed. Some settings calls for multiple spray rounds for protection throughout the entire malaria season.(Day & Africa, 2017)

2.5.3.4 Malaria Vaccine

The World Health Organization recommended further evaluation of RTS, S / AS01 (RTS, S) in a series of pilot implementations in January 2016 (Olotu et al., 2016). This needs to close some knowledge gaps before considering broader deployments at the national level. In January 2016, as part of the Malaria Vaccine Implementation Program, WHO recommended the Malaria Vaccines RTS, S for pilot introduction in selected regions of three African countries: Ghana, Kenya and Malawi. (World Health Organization, 2019) The data obtained from the pilot introduction show that the vaccine has a favourable safety profile. Greatly reduces severe life-threatening malaria. And even during a pandemic, it can be effectively administered in a real childhood vaccination setting. (World Health Organization, 2019) On October 6, 2021, the WHO recommended the use of RTS, S malaria vaccines for the prevention of P. falciparum

malaria in children living in moderately and highly infectious areas. (World Health Organization, 2019).

2.6 Malaria Elimination

As described by Cheng *et al*, 2015 elimination refers to interfering indigenous mosquito-borne malaria spread within a demarcated locality, zero recorded prevalence of locally contracted cases, although new cases brought from other regions will continue to occur. The WHO is committed to eliminating malaria and has reported a tremendous number of countries are working towards elimination of malaria.(World Health Organization, 2015) Previously, about 13 countries have recorded less than 1000 malaria cases in 2000; recently 33 nations are projected to achieve this landmark in 2015. Consequently, in the European region nil malaria cases have been reported by 2015 this depicts success of the Tashkent Declaration to eliminate malaria from the region by 2015 (World Health Organization, 2015).

2.6.1 WHO Malaria Strategy

WHO's global technical strategy for malaria, 2016-2030

This strategy was approved in May 2015 by the World Health organization, being an outline that would guide countries towards controlling and eliminating malaria. The agreed motivated goals for 2030, included decreasing malaria mortality and prevalence by not less than 90%, malaria elimination in not less than 35 countries, and preventing malaria restoration in malaria free countries.(WHO, 2017)

Moreover, they set Interim markers to track the progress, the pro term 2020 targets to sensitize 40% cutbacks in malaria incidence, prevalence and mortality rates and for the eradication of malaria in not less than 10 countries. Not more than half of the world's 91 nations with malaria spread have shown focus in the road to achieve these goals (WHO, 2017) Consequently, to realize these goals the all malaria-affected countries and their

development partners are called upon by the WHO to close the gap in coverage of LLITNS and IRS (WHO, 2017)

2.6.2 Challenges and Way Forward

The fight against malaria has been threatened by quite a number of factors i.e. the rise and spread of mosquito species resistant to insecticides. Since 2010, about 60 countries have reported incidences of resistance to at least one insecticide class used in IRS or ITNs; predominantly of pyrethroid resistance. (WHO, 2017)

Moreover, field diagnosis of malaria in the past have been done using microscopy and rapid diagnostic tests (RDT), that target genus-specific aldolase and lactate dehydrogenase enzymes, the majority of them being specific for the P. falciparum histidine-rich protein –2 (Pf HRP-2). (World Health Organization, 2015)

Recently, some *Plasmodium falciparum* parasites in parts of South America and Africa have deleted the hrp-2 gene. This has brought into question the use of the HRP-2 based RDT tests due to potential false negatives(Lucchi et al., 2013)

Consequently, Expert microscopy similarly has recorded 100 % sensitivity while doing parasite quantification where it has shown detection of as low as 50parasites/µL (WHO, 2014). however, the disadvantage being it's laborious and thus sensitivity goes down significantly. The malaria elimination success therefore, has shown to be achieved through accurate diagnosis followed by correct treatment of all positive confirmed cases.(World Health Organization, 2015)

This calls for highly sensitive diagnostic tools. Furthermore, reports made Africa and Asia states that molecular techniques are needed to compliment microscopy in the quest to eliminate malaria (WHO, 2014)

Moreover, Molecular tools have shown more sensitivity in detecting low parasite infections and correctly identifying the species of malaria parasite. Molecular tools for

malaria diagnosis range from conventional PCR-based assays, real-time PCR assays and, isothermal amplification assays(PMI, 2016)

Therefore, during large scale field application it's paramount to consider cost, robustness, and ease of use. High throughput loop-mediated isothermal amplification in filed application has demonstrated such characteristics (Mccreesh P. 2015)

Additionally, High throughput LAMP has shown a prospective to aid in the fight towards malaria elimination. It differs from PCR in that, it uses a thermal stable enzyme *Bacillus stearothermophilus* that does not need repeated temperature changes that are seen in PCR. This characteristic helps adoption of this method in field evaluations(Mccreesh P. 2015).

Secondly, LAMP end product detection can be done visually checking for the establishment of magnesium pyrophosphate precipitate(Hayashida et al., 2017).Moreover, metal ion indicators, such as calcein, hydroxynaphthol blue and pico-green has been used to detect end point, in addition melt curve analysis, a bioluminescent output in real time (BART), a lateral flow dipstick and a moveable fluorescence detection unit have also been used in real time (Oriero et al., 2015).

Additionally, in the goal to eliminate malaria the WHO projects that those scientific developments and inventions in novel methods, development of new insect control intrusions, improved diagnostics and more effective antimalarial medicines will contribute towards malaria elimination quest, and thus its calling for increased investment in the development and deployment of innovative tools – a critical strategy for reaching global malaria targets.(WHO, 2017)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study samples were collected from Busia County situated at the western region of Kenya. The population of Busia was estimated to be 893,681 (census 2019). According to a census conducted in 2019, Busia County receives an annual rainfall of between 760mm and 2000 mm. majority of this rain occurs during the long rain season between late March and late May, while about 25% falls during the short rains between August and October. The annual temperatures range between 14ºC and 26°C in this area. This county is a malaria endemic region with suitable breeding sites for the Anopheles mosquito vectors that transmit the malaria parasites.

The County borders three other counties which include: Bungoma to the north, Kakamega to the east and Siaya to the south west. Most parts of Busia County fall within the Lake Victoria Basin. The altitude is undulating and rises from about 1,130m above sea level at the shores of Lake Victoria to a maximum of about 1,500m in the Samia and North Teso Hills. The central part of the county, especially Butµla and Nambale Sub-counties, are occupied by a pene plain marked by low flat divides of approximately uniform height, often capped by lateritic and a shallowly incised swampy drainage system. This provides breeding grounds for vectors that cause malaria. The samples were collected during the peak malaria transmission season from August to November 2016.

3.2 Study Population

The current study utilized archived DBS samples from a previous drug efficacy testing study (KEMRI SSC 2276). The study population of that study comprised children between the ages of 6 months and 12yrs from Matayos, Busia County who presented

with acute, symptomatic, uncomplicated *P. falciparum* malaria. Blood samples were collected from these children during the peak malaria transmission season from August to November 2016. From this whole blood samples DBS were prepared and archived. Children who tested positive for *Plasmodium falciparum* were put on Artemether-Lumefantrine and Dihydroartemisinin and followed up for 42 days testing for drug therapeutic efficacy testing. For the current study, 134 DBS were selected by simple random sampling from a total of 302 archived DBS samples collected from baseline and the first two days post treatment. The goal was to select potentially low parasitaemia infections for evaluation using HT LAMP. These would be samples which were determined to be positive for parasites at baseline by microscopy but subsequent present as negative by microscopy immediately after drug treatment.

3.2.1 Inclusion Criteria

Integrity and storage of the archived samples was checked and samples that had no moulds and dry DBS were included in the current study.

3.2.2 Exclusion Criteria

Integrity and storage of the archived samples was checked and samples that had moulds and wet DBS due to poor storage were excluded from the current study.

3.3 Sample Size Determination

The sample size was calculated using the formula designed by Karimollah Hajian-Tilaki for sample size estimation in diagnostic test studies

Sensitivity 89.7% (Hayashida et al., 2017)

Statistical significance 0.05

Prevalence 27% (NMEP 2016)

$$N = \frac{Z2a/2\ se\ (1-se)}{d2\ (1-P)}$$ (Hajian-Tilaki, 2014)

Where:

N = sample size,

$Z^2a/2$= 1.96

Se= sensitivity 0.897

d^2 = margin of error, 0.05

p= prevalence 0.27

$$N = \frac{(1.962) \times 0.897(1-0.897)}{0.052 \times (1-0.27)}$$

This translates into N = **103**

3.4 Optimizing of High Throughput LAMP Assay

3.4.1 Extraction of plasmodium falciparum culture using Chelex method

Plasmodium culture of the strain D6 was used in extraction. A dried blood spot was made using a drop of blood. DNA was then extracted from the DBS. Briefly; a 6 mm filter paper disc punches were cut using a sterile scalpel blade. This disc was incubated in 100 µl 0.5 % Saponin in PBS for 12 hours at 4° C. The resulting brown solution was discarded and substituted with 100 µl 1 × PBS refrigerated at 4° C for 20 minutes. The solution was then discarded and refilled with100µl of DNase free water and 50µl of 20% Chelex consecutively. The tubes were heated at 98°C and vortexed every two minutes and repeated up to 5 times on a block heater. The solution was then centrifuged for 2

min at 4000 rpm and 100 µL of the supernatant liquated into 200µL Eppendorf tubes and stored at −20° C for PCR analysis.(Musapa et al., 2013)

3.4.2 Preparation of LAMP Assay

The setup of LAMP reagents followed the process as described by Hayashida *et al* (2017) with minor alterations. Briefly, 35 nmol each of dNTPs mix and 8 U of Bst2.0 WS DNA polymerase was aliquoted on the lids of the 8-strip PCR tubes. The Indicator made up of 3.5 nl SYBR green, 3.2 pmol of FIP and BIP, 0.4 pmol of F3 and B3, and 1.6 pmol of LF and LB primers were aliquoted in the wells of 8-strip PCR tubes. (Hayashida et al., 2017) The primers are indicated on table 1 below as previously described by (Polley et al., 2010) targeting mitochondria DNA.

Table 3.1: Oligonucleotide primers used in HT LAMP and their sequences

Primer type	Sequence
FIP	CAGTATATTGATATTGCGTGACGACCTTGCAATAAATAATATCTAGCGTGT
BIP	AACTCCAGGCGTTAACCTGTAATGATCTTTACGTTAAGGGC
F3	TATTGGCACCTCCATGTCG
B3	AACATTTTTTAGTCCCATGCTAA
LF	GTGTACAAGGCAACAATACACG
LB	GTTGAGATGGAAACAGCCGG

3.4.3 Testing of LAMP Assay Using a Positive Control and DMSO

P. falciparum culture of 550p/µl (9890ng /uL of DNA) was extracted using chelex method and gave a yield of (9890ng /uL of DNA). This was used to optimize and validate the HT LAMP assay. 10 fold Serial dilutions of the DNA were made (McCreeshP. 2015) Various concentrations of DMSO were prepared i.e. 1%, 2%, 3% and 4%. 1µL of positive control plus 1µl of DMSO were added to make a 25µl reaction mix, the PCR tube was then mixed thoroughly to ensure adequate mixing of reagents. The water bath was then set at 63°C and the tubes incubated for 1 hour. Amplified DNA

products were visualized using syber green by use of a trans illuminator. (Hayashida et al., 2017)

3.5 Nested PCR on archived field samples

The Nested PCR reaction was done targeting *P. falciparum* 18s rRNA gene, it was done through two stages i.e. Outer and Nested PCR

3.5.1 Outer PCR on archived filed samples

The outer PCR reaction mixture was as described in table 3.0. The cycling conditions were set at 98°C for initial denaturation for 4 minutes followed by denaturation at 94°C for 1 min, annealing at 65°C for 2 minutes and extension at 72°C for 2 minutes and 72^{o} C for 4 minutes. These was repeated for 30 cycles and the products stored at -20^{o} C. (Echeverry et al., 2016)

3.5.2 Nested PCR on outer PCR products

Outer PCR products formed template for Nested PCR, the reaction mixture was as described in table 2 below. The thermal cycling conditions was 94°C for 4 minutes initial denaturation followed by denaturation at 94 ° C for 30 seconds followed by annealing at 62° C for 1 minute and extension at 72° C for 1minute. These steps were repeated for 35 cycles and then followed by a final extension at 72° C for 1 minute and 72° C for 4 minutes.(Echeverry et al., 2016)

Table 3.2: Nested PCR reaction Table (Echeverry et al., 2016)

Outer PCR Reactions	Nested PCR Reactions
Template DNA	Template DNA
2.5mM Magnesium chloride	2.5mM Magnesium chloride
100 nM dNTP	100 nM Dntp
100 nM RPLU1	100 nM RPLU3
100 nM RPLU5	100 nM RPLU4
1 unit Taq polymerase	1 unit Taq polymerase
1X PCR Buffer	1X PCR Buffer
PCR water	PCR water

Table 3.3: Oligonucleotide primers used in Nested PCR and their sequences (Echeverry et al., 2016)

Primer type	Sequence
fPLU1	TCAAAGATTAAGCCATGCAAGTGA
rPLU3	TTTTTATAAGGATAACTACGGAAAAGCTGT
fPLU4	TACCCGTCATAGCCATGTTAGGCCAATACC
rPLU5:	CCTGTTGTTGCCTTAAACTTC

3.5.3 PCR Products Visualization by Gel Electrophoresis

Gel electrophoresis is a technique that is used to demonstrate DNA amplified after PCR, this was done by preparing 1.5% agarose gel with ethidium bromide as a fluorescent stain. 1.5 grams of agarose was weighed and dissolved by heating in 100ml of TAE buffer. It was allowed to set and then submerged in TAE buffer in the electrophoretic tank. The electrophoretic tank set for 30 minutes at 80 volts. Visualization of amplified DNA was done using ultraviolet light against a 100 base pair DNA ladder and the result's documented using a camera.

3.6 (HT LAMP) Reaction on archived samples

The pre setup 8 strip was used to perform HT LAMP assay on DNA from the archived malaria samples as described by (Britton, Cheng, Sutherland, & McCarthy, 2015).

Reactions were done in 25 µL final volumes made by adding 1µl of extracted DNA and 1µl of DMSO the tubes were mixed thoroughly to mix the reagents. A water bath was set at 63°C and the plate Incubated for 1 hour, the amplified DNA products were visualized using SYBR green using a trans illuminator, Positive samples showed a bright fluorescent green colour under a trans illuminator.

3.7 Data management and statistical analysis

Activities done in the laboratory were captured in the laboratory work book. The results were entered in Microsoft excel stored in a secure non-networked environment. The sensitivity, specificity, positive predictive value and negative predictive value of HT LAMP was calculated and the extent of agreement with Nested PCR and Microscopy compared using Kappa statistics.

3.8 Ethical Considerations

This study used archived samples to optimize and evaluate the Assay (protocol SCC 2276), permission was obtained from the Principal Investigator of the study to use the archived samples. Ethical approval was also granted by Scientific Steering Committee and Ethical Review Committee of the Kenya Medical Research Institute (No. KEMRI/RES/7/3/1).

3.9 Expected Output

The findings of this study were published in a peer-reviewed journal enabling the PI to attain a Master's degree.

CHAPTER FOUR

RESULTS

4.1 LAMP assay optimization and Evaluation

4.1.1 Optimization of Primer Ratio

D6 strain of *plasmodium falciparum* with 550 parasites per microliter of blood (9890ng /uL of DNA) was used to standardize the assay. DNA was extracted using Chelex method of extraction. The primer ratio that showed optimum amplification was 8:1, ratio of the inner primers to outer primers , the choice of primer ratio was guided by a study done in Kenya (Mugambi et al., 2015).

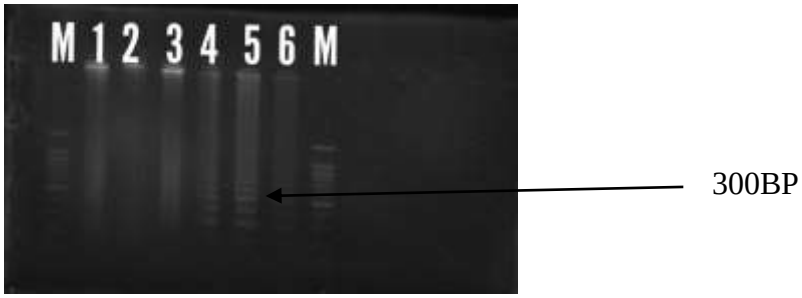


Figure 4.1: Primer ratio optimization was done

The 1.5 % agarose gel plate shows amplification of *plasmodium falciparum* with variation of inner to outer primer ratio. Lane 1 - 1:1, Lane 2 – 2:1, Lane 3 – 4:1, Lane 4 – 6:1. Lane 5– 8:1 Lane 6 – 10:1. 8:1 ratio readily amplified DNA, setting precedence for the HT LAMP technique

4.1.2 Temperature optimization

Various Temperatures were optimized and the optimum temperature that gave amplification was 63^{o} C. this is as deduced in the figure below

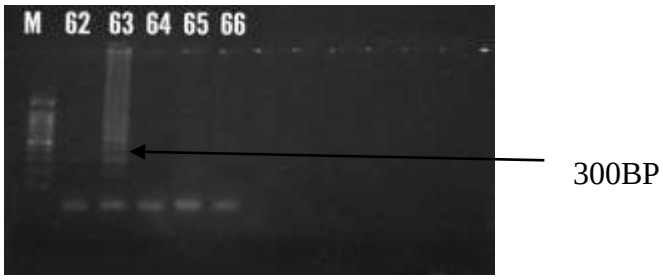


Figure 4.2: Temperature optimization

Optimization of temperature conditions was confirmed on 1.5% agarose gel as shown in lanes 1, 2, to 5. Assessment was based on the gel electrophoresis analysis of the LAMP products: M-100 bp ladder, lanes 1 to 5 show the tested temperature range of 62–66 °C, and the maximum amplification (ladder-like pattern) was observed at 63 °C as indicated in the figure above.

4.1.3 Time optimization

The optimum time that gave amplification was found to be 45minutes as indicated in the table below:

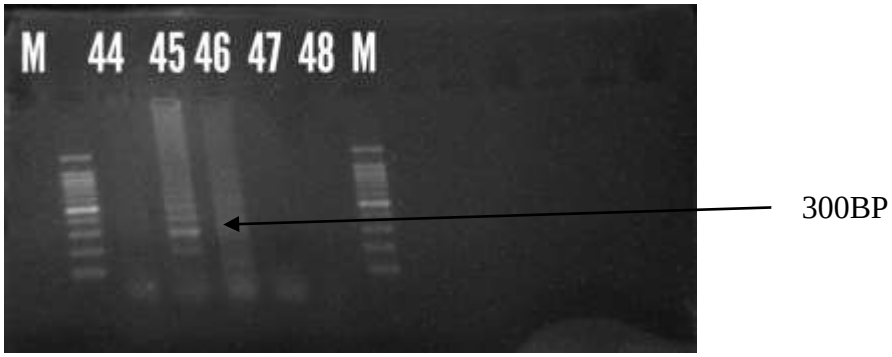


Figure 4.3: Time optimization

The 1.5 % agarose gel plate shows amplification of *plasmodium falciparum* at certain range of times.

45 minutes gave the best amplification and hence adopted for the assay.

4.1.4 DNA polymerase Enzyme optimization

Bacillus stearothermophilus DNA polymerase was used in the LAMP technique as it produced results under the above-mentioned conditions. One unit of *Bst* DNA polymerase requiring an optimum temperature of 63^{o} C to incorporate 35 nmol of dNTPs into polynucleotide fraction in 45 min.

4.1.5 Sensitivity and Specificity in varied DNA Concentrations with addition of DMSO

The primers were adapted without modifications from the *P. falciparum* malaria CZC-LAMP assay developed by (Hayashida et al., 2017). The LAMP primer sets were designed for mitochondria DNAs of *Plasmodium falciparum* (Pf) and human-infective species other than Pf (non-Pf; P. vivax, P. ovale, *P. malariae*. They were evaluated and tested using human blood DNA samples from a malaria endemic area in eastern Zambia (Hayashida et al., 2017). This assay was able to amplify serially diluted extracted DNA of *plasmodium falciparum* species. This was confirmed by visualization under gel electrophoresis and compared to Nested PCR. A 10-fold serial dilution was done of the known standard and 4% of DMSO was added to enhance sensitivity. The limit of detection of the assay was determined to be 5 parasites per microliter equivalent to 98.9 ng/ uL DNA.

Figure 4.4: UV light detection of HT LAMP

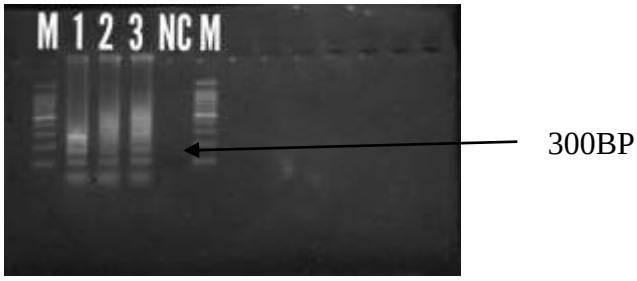


Figure 4.5: Gel Visualization of HT LAMP

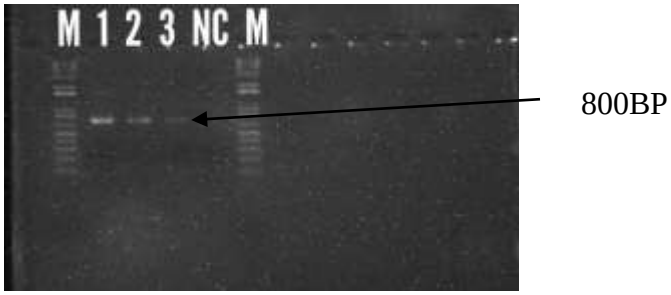


Figure 4.6: Gel Visualization of Nested PCR

Sample 1 had 9890ng /uL of DNA, sample 2 had 989ng /uL of DNA and sample 3 had 98. 9ng /uL of DNA. NC is the negative control while M is the molecular ladder of 100 base pairs.

4.2 Evaluation of HT LAMP assay to Microscopy

LAMP PCR was done on 134 archived field samples then compared to Microscopy as deduced in the tables below.

Table 4.1: Contingency Table Comparing Microscopy and HT LAMP PCR

		HT Lamp PCR		
Microscopy		**Positive**	**Negative**	**Total**
	Positive	33	0	**33**
	Negative	10	91	**101**
Total		**43**	**91**	**134**

Both assays detected 33 samples as positive, 10 samples were positive in HT LAMP PCR and negative in Microscopy. The assays agreed that 91 samples were negative and thus the sensitivity, specificity, positive predictive value and negative predictive value was as below:

Table 4.2: Performance characteristics of HT LAMP PCR against Microscopy

Sensitivity	76.7%
Specificity	100 %
Positive predictive value	100%
Negative predictive value	91%

Table 4.3: Kappa Agreement of Microscopy and HT LAMP PCR

	Value	**Asymp. Std. Error[a]**	**Approx. T[b]**	**Approx. Sig.**
Measure of Agreement Kappa	**.818**	.055	9.626	.000
N of Valid Cases	134			

Cohen's κ was run to determine if there was agreement between two tests for diagnosing malaria on 134 patients. There was a significant agreement between HT LAMP PCR and microscopy, $\kappa = 0.818$, $p < .0001$.

4.3 Evaluation of Nested PCR to Microscopy

Nested PCR was done on 134 samples and compared to Microscopy as described in table 4.4

Table 4.4: Contingency Table Comparing Microscopy and NESTED PCR

		Nested PCR		
Microscopy		**Positive**	**Negative**	**Total**
	Positive	33	0	**33**
	Negative	13	88	**101**
Total		**46**	**88**	**134**

Both assays detected 33 samples as positive, 13 samples were positive in NESTED PCR and negative in Microscopy. The assays agreed that 88 samples were negative and thus the sensitivity, specificity, positive predictive value and negative predictive value were as in table 4.5.

Table 4.5: Performance characteristics of NESTED PCR against Microscopy

Sensitivity	71.7%
Specificity	100 %
Positive predictive value	100%
Negative predictive value	87.1%

Table 4.6: Kappa Agreement of Microscopy and NESTED PCR

	Value	**Asymp. Std. Error[a]**	**Approx. T[b]**	**Approx. Sig.**
Measure of Agreement Kappa	.769	.059	9.152	.000
N of Valid Cases	134			

Cohen's κ was run to determine if there was agreement between two tests for diagnosing malaria on 134 patients. There was a strong agreement statistically between Nested PCR and Microscopy, $\kappa = .769$, $p < .0001$.

4.4 Evaluation of LAMP assay to Nested PCR

The optimized and evaluated LAMP assay was evaluated using 134 archived field samples and the results compared to Nested PCR as illustrated in the figures and tables below.

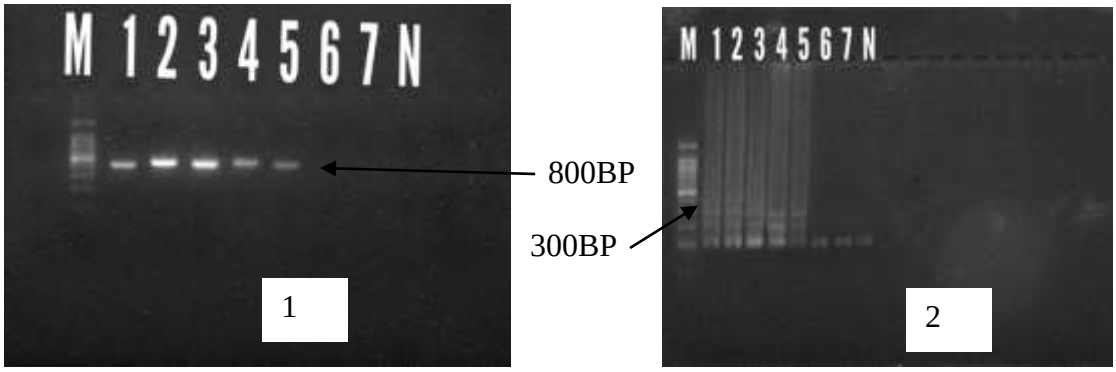


Figure 4.7: Nested PCR agarose gel picture 1 and HT LAMP agarose gel picture 2

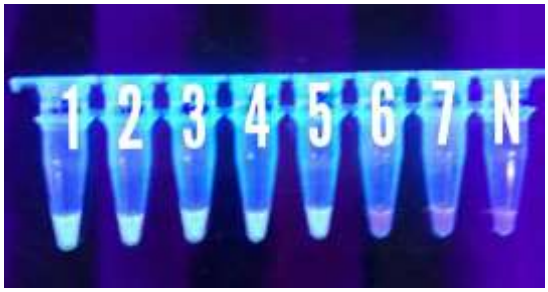


Figure 4.8: Visual detection of LAMP under UV light

Table 4.7: Contingency Table Comparing HT LAMP Assay and NESTED PCR

		HT Lamp		
Nested PCR		**Positive**	**Negative**	**Total**
	Positive	43	0	**43**
	Negative	3	88	**91**
Total		**46**	**88**	**134**

Both assays detected 43 samples as positive, 3 sample were positive in Nested PCR and negative in LAMP PCR. The assays agreed that 88 samples were negative and thus the sensitivity, specificity, positive predictive value and negative predictive value was calculated as in table 4.8

Table 4.8: Performance characteristics of Nested PCR against HT LAMP PCR

Sensitivity	93.5%
Specificity	100 %
Positive predictive value	100%
Negative predictive value	96.7%

Table 4.9: Kappa Agreement of HT LAMP Assay and Nested PCR

	Value	**Asymp. Std. Error[a]**	**Approx. T[b]**	**Approx. Sig.**
Measure of Agreement Kappa	.950	.029	11.006	.000
N of Valid Cases	134			

Cohen's κ was run to determine if there was agreement between two tests for diagnosing malaria on 134 patients. There was a strong agreement between the PCR and LAMP, $\kappa = .943$, $p < .0001$.

CHAPTER FIVE

DISCUSSION

The directive by WHO geared to malaria elimination, will only be realised if there are highly sensitive diagnostic tools that will be able to detect asymptomatic malaria infections. (The malERA Consultative Group on Diagnoses, 2011).

In this project, i optimized and evaluated an assay that was able to amplify DNA at a primer ratio of 8:1 between the inner and outer primers. It worked best at temperatures of 63^{o} Celsius for 45 minutes. This was consistent with a study done in Kenya where the temperatures were optimized at 63^{o} Celsius for 45 minutes (Mugambi et al., 2015). Additionally, the assay has a low detection threshold of 5 parasites per µl of blood (98.9ng /uL of DNA). It has high throughput capacity hence many samples can potentially be handled at the same time. The optimized and evaluated HT LAMP PCR was able to identify 43 samples as positive, among which ten were negative in microscopy. In addition, both diagnostic methods agreed that 88 samples were negative. This meant that the three negative samples in Microscopy had a low parasite count that could have been difficult to pick under microscopy. However, Kappa comparison gave a strong agreement between the two diagnostic methods indicating that both methods can be relied on for malaria diagnosis. In general, HT LAMP PCR being a molecular technique and with a low parasite detection threshold was able to pick the parasites and hence demonstrated higher sensitivity. This is consistent with previous studies which have demonstrated HT LAMP PCR as a cheap and sensitive tool for malaria diagnosis (Tambo et al., 2018)

NESTED PCR identified 46 samples as positive among which thirteen samples were negative in microscopy. Moreover, both diagnostic methods agreed that 88 samples were negative. This demonstrates a higher sensitivity of Nested PCR to low parasite counts that were missed in microscopy. This consistently agrees with a study done in Southern

Ethiopia where Nested PCR demonstrated higher sensitivity than microscopy (Mekonnen et al., 2014).

Comparison between HT LAMP PCR and Nested PCR demonstrated that Nested PCR had superior diagnostic capability than LAMP PCR. This was deduced as a result of Nested PCR detecting 46 samples as positive, 3 of which read negative in HT LAMP PCR. However, Kappa comparison gave a strong agreement indicating that both molecular diagnostic methods would be relied in diagnosis of malaria parasites. These results are in consistent with the study done in Sabah, Malaysia that demonstrated Nested PCR as superior in diagnosis compared to HT LAMP(Lau et al., 2016). In addition, the turnaround time of getting results in HT LAMP was an 8th of the turnaround time for Nested PCR. HT LAMP PCR has a basic benchtop preparation process that makes it an appropriate diagnostic tool, even for low resource settings in health facilities, compared to Nested PCR which requires working on ice and a PCR cabinet to avoid contamination.(Tambo et al., 2018)

Additionally, HT LAMP PCR has a number of pluses over the existing molecular diagnostic methods. The *Bacillus steothermophillus polymerase* enzyme that catalyzes the LAMP reaction is more robust with respect to inhibition than *Taq* polymerase enzyme (Notomi et al., 2000).

It is therefore possible to use a simple, prompt, and low-cost procedure for sample preparation, in contrast to that required for Nested PCR (Polley et al., 2010). The ease of preparing the samples and the prompt reaction results with a simple visible endpoint makes the assay more ideal for application in field settings. For instance, detection of amplification products with ultraviolet light at the end of the incubation time, provided visual comparison of a positive test from a negative test eliminating the need for electrophoresis thus reducing cost. SYBR green, a DNA intercalating dye that turns green for positive results and color remains orange in a negative result.

A water bath was able to provide isothermal conditions and hence demonstrating the feasibility of using this assay under field conditions not only for its simplicity but also for cost effectiveness in resource poor settings where malaria is endemic.(Hartmeyer et al., 2019).

Majority of the identified costs in the application of LAMP technique went into buying molecular reagents such as *Bacillus steothermophillus* DNA polymerase, SYBR green DNA intercalating dye, dinucleotides, primers, dimethylsulphoxide, which were bought at a market price of (USD) 1000. They were estimated to run about 50 reactions. This translates into approximately (USD) 20 for analyzing one sample. This is ten times higher than microscopy which cost on average (USD) 0.2. This is however relatively cheaper than when using Nested PCR as expensive equipment will be needed.

5.1 Study Limitation

Limited resources posed a challenge to completion of the project.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

I optimized and evaluated an assay with similar performance characteristics to Nested PCR, it is less expensive, easy to use, and amicable to large scale-surveillance studies in developing country settings. Upon its adoption in diagnosis it will help in detection of low *plasmodium falciparum* infections that act as reservoirs of malaria disease. This will complement malaria elimination efforts.

6.2 Recommendations

1. To Lyophilize the HT LAMP reagents to make the assay more user friendly in the field set up.
2. Evaluate HT LAMP assay with adult samples to help diagnose low parasite intensity *plasmodium falciparum* infections.
3. Improvement of the assay to a multiplex assay that will be able to screen other *plasmodium* species.
4. To incorporate more quick DNA extraction methods for ease field applicability.

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APPENDICES

Appendix I: Results of Malaria Diagnostic Tests for Archived Extracted DNA of Blood Samples Collected from Busia, Western Kenya

Serial no.	Archived Sample No.	Day sample collected	Microscopy Parasitemia	Nested PCR	HT LAMP
1.	001	Day 0	66720	+	+
2.	002	Day 0	12000	+	+
3.	003	Day 0	2440	+	+
4.	004	Day 0	1200	+	+
5.	005	Day 0	20400	+	+
6.	006	Day 0	2240	+	+
7.	007	Day 0	49440	+	+
8.	008	Day 0	12440	+	+
9.	009	Day 0	2840	+	+
10.	010	Day 0	29760	+	+
11.	011	Day 0	55040	+	+
12.	012	Day 0	4040	+	+
13.	013	Day 0	43540	+	+
14.	002	Day 1	400	+	+
15.	003	Day 1	200	+	+
16.	004	Day 1	480	+	+
17.	005	Day 1	356	+	+
18.	006	Day 1	1080	+	+
19.	009	Day 1	0	-	-
20.	012	Day 1	0	-	-
21.	018	Day 1	0	-	-
22.	021	Day 1	0	-	-
23.	022	Day 1	3160	+	+
24.	002	Day 2	0	-	-
25.	003	Day 2	0	-	-
26.	004	Day 2	0	-	-
27.	005	Day 2	0	+	+
28.	006	Day 2	0	-	-
29.	007	Day 2	0	-	-
30.	008	Day 2	0	-	-
31.	009	Day 2	0	-	-
32.	010	Day 2	0	-	-
33.	011	Day 2	0	-	-
34.	012	Day 2	0	-	-

35.	013	Day 2	0	-	-
36.	014	Day 2	0	-	-
37.	015	Day 2	0	-	-
38.	016	Day 2	0	-	-
39.	017	Day 2	0	-	-
40.	018	Day 2	0	-	-
41.	019	Day 2	0	-	-
42.	020	Day 2	0	-	-
43.	021	Day 2	0	-	-
44.	022	Day 2	0	-	-
45.	023	Day 2	0	-	-
46.	024	Day 2	0	-	-
47.	025	Day 2	0	-	-
48.	026	Day 2	0	-	-
49.	027	Day 2	0	-	-
50.	028	Day 2	0	-	-
51.	029	Day 2	0	-	-
52.	030	Day 2	0	-	-
53.	031	Day 2	0	-	-
54.	032	Day 2	0	-	-
55.	033	Day 2	0	-	-
56.	034	Day 2	0	-	-
57.	035	Day 2	0	+	+
58.	036	Day 2	0	-	-
59.	037	Day 2	0	-	-
60.	038	Day 2	0	+	+
61.	039	Day 2	0	+	+
62.	040	Day 2	0	+	+
63.	041	Day 2	0	+	+
64.	042	Day 2	0	+	+
65.	043	Day 2	0	+	+
66.	044	Day 2	0	-	-
67.	053	Day 1	1560	+	+
68.	054	Day 1	1120	+	+
69.	055	Day 1	1400	+	+
70.	056	Day 1	1200	+	+
71.	057	Day 1	0	-	-
72.	058	Day 1	0	-	-
73.	059	Day 1	0	-	-
74.	045	Day 2	0	-	-
75.	046	Day 2	0	-	-
76.	047	Day 2	0	-	-

77.	048	Day 2	0	+	-
78.	049	Day 2	0	+	+
79.	050	Day 2	0	-	-
80.	051	Day 2	0	-	-
81.	052	Day 2	0	-	-
82.	053	Day 2	0	-	-
83.	054	Day 2	0	+	-
84.	055	Day 2	0	-	-
85.	056	Day 2	0	-	-
86.	057	Day 2	0	+	-
87.	058	Day 2	0	-	-
88.	078	Day 2	2240	+	+
89.	059	Day 2	0	-	-
90.	060	Day 2	0	-	-
91.	061	Day 2	0	-	-
92.	062	Day 2	0	-	-
93.	063	Day 2	0	-	-
94.	064	Day 2	0	-	-
95.	065	Day 2	0	-	-
96.	066	Day 2	0	-	-
97.	067	Day 2	0	-	-
98.	068	Day 2	0	-	-
99.	059	Day 1	1000	+	+
100.	060	Day 1	1500	+	+
101.	061	Day 1	1640	+	+
102.	062	Day 1	1469	+	+
103.	063	Day 1	1600	+	+
104.	064	Day 1	1160	+	+
105.	065	Day 1	1320	+	+
106.	066	Day 1	2040	+	+
107.	069	Day 2	0	-	-
108.	070	Day 2	0	-	-
109.	071	Day 2	0	-	-
110.	072	Day 2	0	-	-
111.	073	Day 2	0	-	-
112.	074	Day 2	780	+	+
113.	075	Day 2	0	+	+
114.	076	Day 2	0	-	-
115.	077	Day 2	0	-	-
116.	078	Day 2	0	-	-
117.	079	Day 2	0	-	-
118.	080	Day 2	0	-	-

119.	081	Day 2	0	-	-
120.	082	Day 2	0	-	-
121.	083	Day 2	0	-	-
122.	084	Day 2	0	-	-
123.	085	Day 2	0	-	-
124.	086	Day 2	0	-	-
125.	087	Day 2	0	-	-
126.	088	Day 2	0	-	-
127.	089	Day 2	0	-	-
128.	090	Day 2	0	-	-
129.	091	Day 2	0	-	-
130.	092	Day 2	0	-	-
131.	093	Day 2	0	-	-
132.	094	Day 2	0	-	-
133.	095	Day 2	0	-	-
134.	096	Day 2	0	-	-

COLOUR CHART

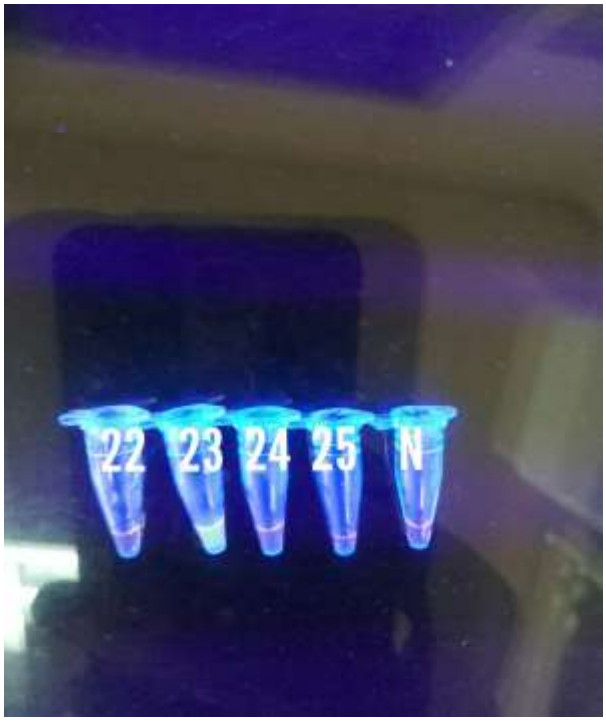
Yellow: Indicated repeated samples in day 1 and day 2

Red: Indicated samples that were negative with microscopy and positive in both HT Lamp and Nested PCR

Green: Indicated samples Negative in HT LAMP and positive in Nested PCR

No Colour: These are results that agreed in all the diagnostic tests

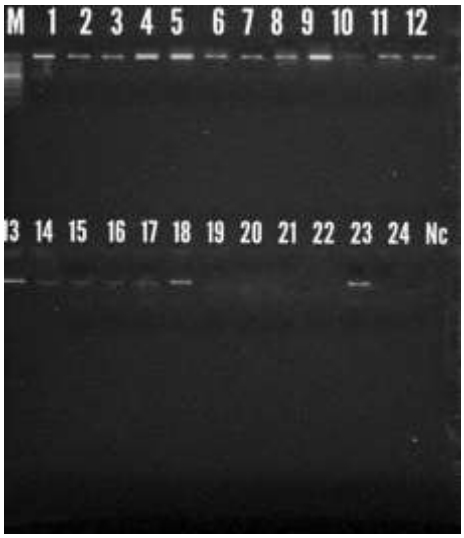
Appendix II: LAMP Pictures on the UV Light

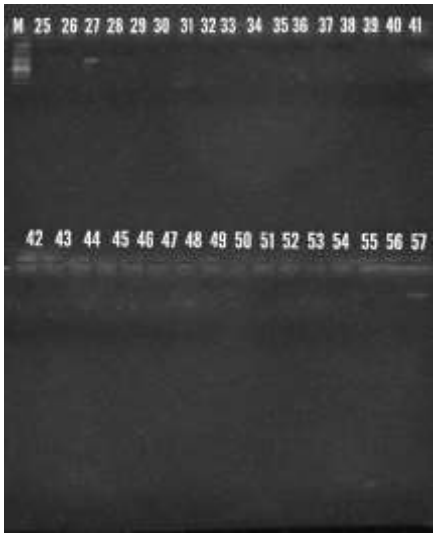



26 27 28 29 N

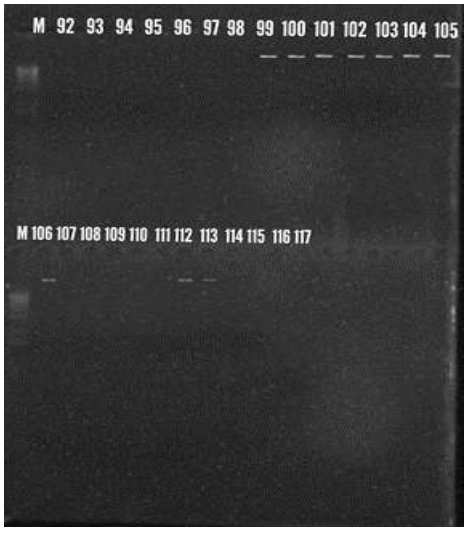

CAUTION
30 31 32 33 34 35 36 N
37 38 39 40 41 42 43 N
44 45 46 47 48 49 50 N
51 52 53 54 55 56 57 N
58 59 60 61 62 63 64 N
65 66 67 68 69 70 71 N
72 73 74 75 76 77 78 N
79 80 81 82 83 84 85 N
86 87 88 89 90 91 92 N
93 94 95 96 97 98 99 N
100 101 102 103 104 105 106 N
107 108 109 110 111 112 113 N
114 115 116 117 118 119 120 N
121 122 123 124 125 126 127 N
128 129 130 131 132 133 134 N

Appendix III: Nested PCR Pictures



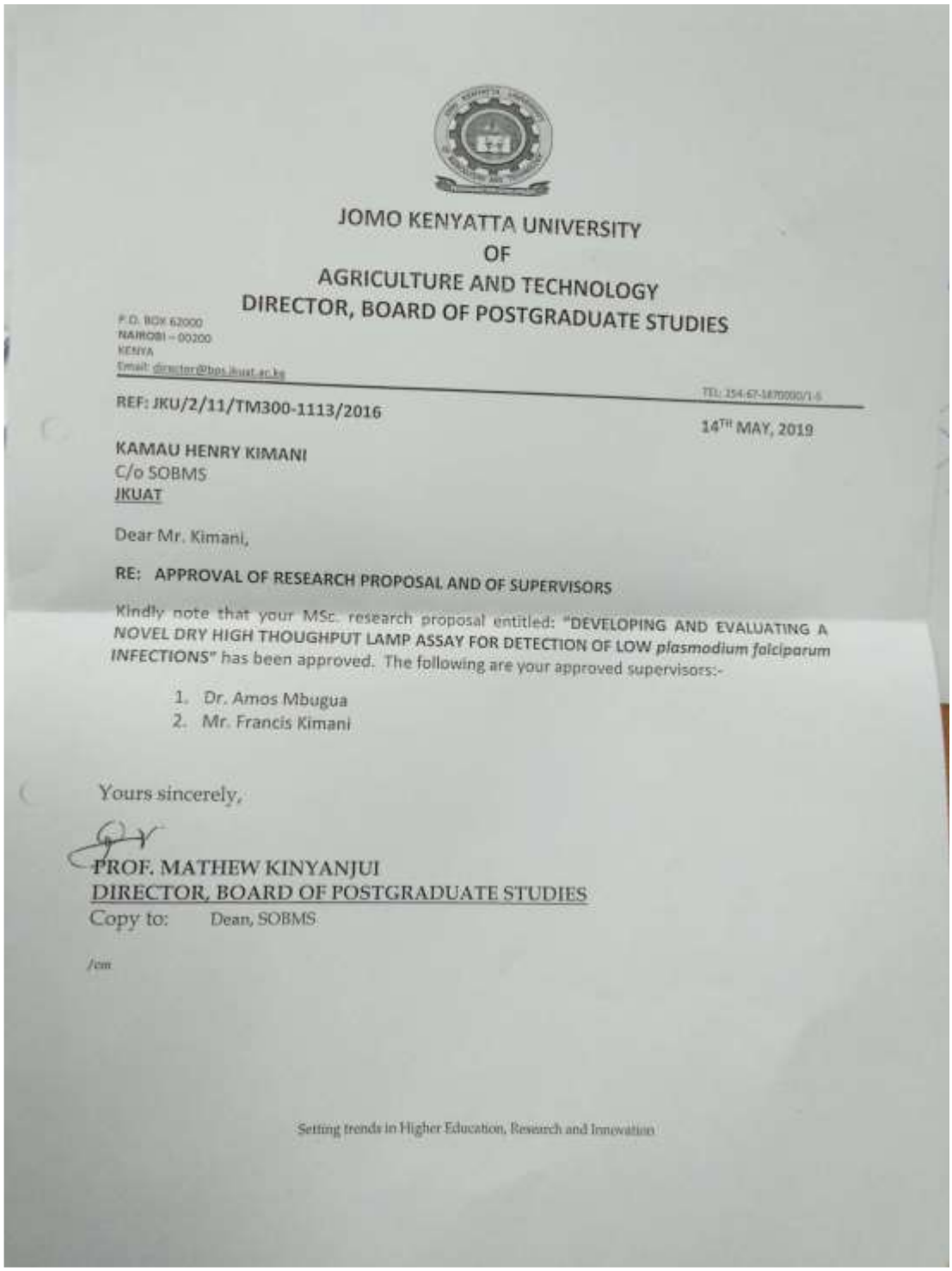



M 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72
73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91


M 92 93 94 95 96 97 98 99 100 101 102 103 104 105
M 106 107 108 109 110 111 112 113 114 115 116 117


118 119 120 121 123 124 125 126 127 128 129 130 131 132 133 134 NC

Appendix IV: Proposal Approval



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REF: JKU/2/11/TM300-1113/2016

14TH MAY, 2019

KAMAU HENRY KIMANI
C/o SOBMS
JKUAT

Dear Mr. Kimani,

RE: APPROVAL OF RESEARCH PROPOSAL AND OF SUPERVISORS

Kindly note that your MSc. research proposal entitled: "DEVELOPING AND EVALUATING A NOVEL DRY HIGH THOUGHPUT LAMP ASSAY FOR DETECTION OF LOW *plasmodium falciparum* INFECTIONS" has been approved. The following are your approved supervisors:-

1. Dr. Amos Mbugua
2. Mr. Francis Kimani

Yours sincerely,

PROF. MATHEW KINYANJUI
DIRECTOR, BOARD OF POSTGRADUATE STUDIES
Copy to: Dean, SOBMS

/cm

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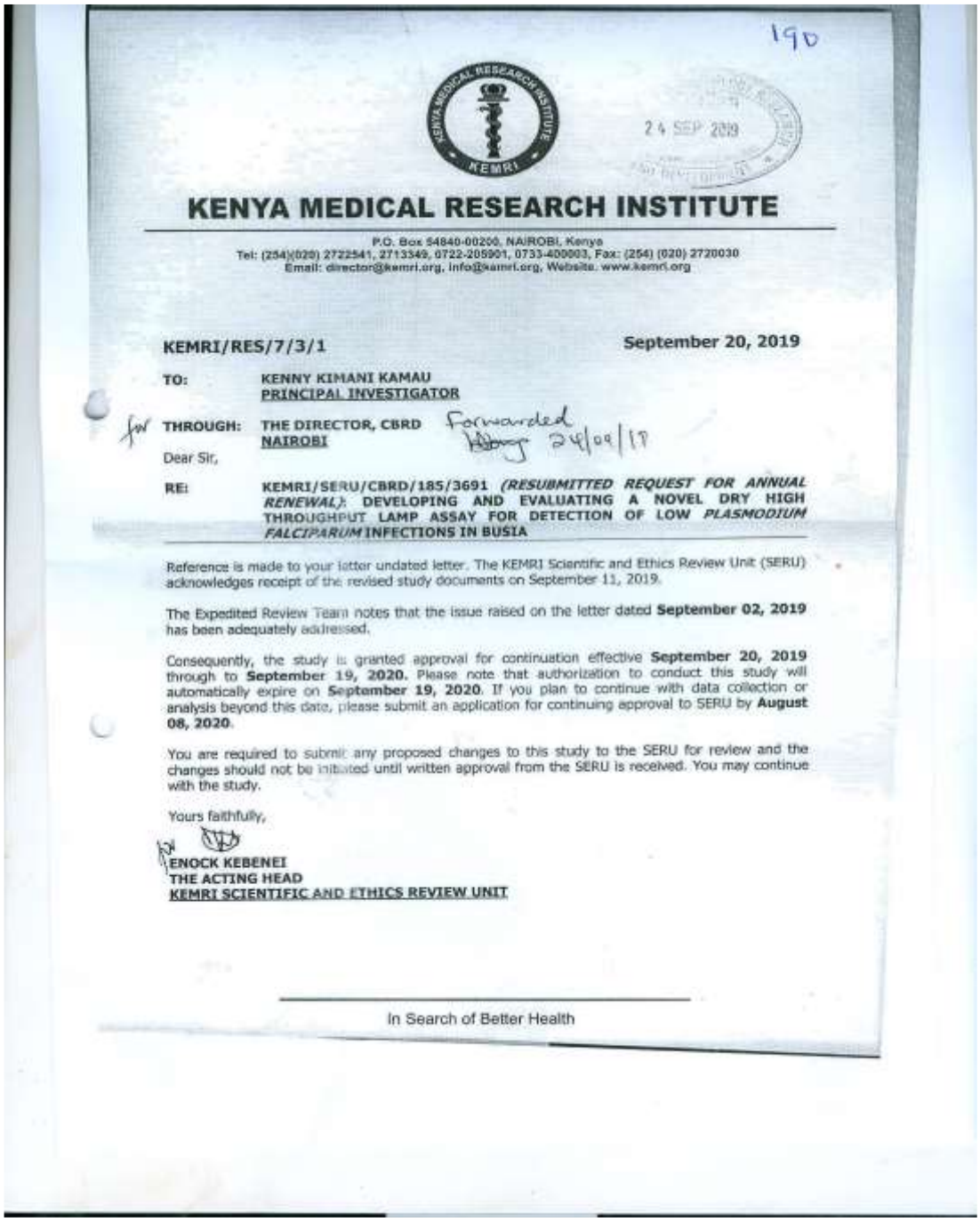
Appendix V: Ethical Approval Letter



190

24 SEP 2019

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KEMRI/RES/7/3/1 **September 20, 2019**

TO: **KENNY KIMANI KAMAU**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CBRD**
NAIROBI

Forwarded 24/09/19

Dear Sir,

RE: **KEMRI/SERU/CBRD/185/3691 *(RESUBMITTED REQUEST FOR ANNUAL RENEWAL)*: DEVELOPING AND EVALUATING A NOVEL DRY HIGH THROUGHPUT LAMP ASSAY FOR DETECTION OF LOW *PLASMODIUM FALCIPARUM* INFECTIONS IN BUSIA**

Reference is made to your letter undated letter. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on September 11, 2019.

The Expedited Review Team notes that the issue raised on the letter dated **September 02, 2019** has been adequately addressed.

Consequently, the study is granted approval for continuation effective **September 20, 2019** through to **September 19, 2020.** Please note that authorization to conduct this study will automatically expire on **September 19, 2020.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to SERU by **August 08, 2020.**

You are required to submit any proposed changes to this study to the SERU for review and the changes should not be initiated until written approval from the SERU is received. You may continue with the study.

Yours faithfully,

ENOCK KEBENEI
THE ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health