EVALUATION OF THE COLORIMETRIC MALACHITE GREEN LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (MG-LAMP) ASSAY FOR THE DETECTION OF *PLASMODIUM* SPECIES AT TWO DIFFERENT HEALTH FACILITIES IN A MALARIA ENDEMIC AREA OF WESTERN KENYA

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Evaluation of the Colorimetric Malachite Green Loop-Mediated Isothermal Amplification (MG-LAMP) Assay for the Detection of *Plasmodium* Species at Two Different Health Facilities in a Malaria Endemic Area of Western Kenya

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DECLARATION

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

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DEDICATION

I dedicate this project to my parents, Dr. Johnson Mwangi Gachugia and Dr. Jane Wangari Macharia, and my brother Collins Macharia for their words of wisdom, encouragement and support during my studies.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ACTs	Artemisinin-based Combination Therapies
CDC	Centre for Disease Control and Prevention
CI	Confidence Interval
DBS	Dried Blood Spots
DNA	Deoxyribonucleic Acid
EDTA	Ethylene diamine tetra-acetic acid
HRP-2	Histidine Rich Protein-2
JICA	Japan International Cooperation Agency
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI/CGHR	Kenya Medical Research Institute/Centre for Global Health Research
KU-ERC	Kenyatta University Ethical Review
MG-LAMP	Malachite Green Loop-mediated Isothermal Amplification
NAATs	Nucleic Acid Amplification Tests
NMCP	National Malaria Control Programme
NPV	Negative Predictive Value
P/µL	Parasite per microlitre
PMI	President's Malaria Initiative
PPV	Positive Predictive Value
QA/QC	Quality assurance/ Quality control;
qPCR	Quantitative Real-Time Polymerase Chain Reaction
QT-NASBA	Quantitative Nucleic Acid Sequence-Based Amplification

RDTs	Rapid diagnostic tests
WHO	World Health Organization

ABSTRACT

Prompt diagnosis and effective malaria treatment are key strategies in the control of malaria. However, the recommended routine diagnostic methods, particularly microscopy and rapid diagnostic tests (RDTs), do not have robust quality assurance systems in malaria endemic areas. This study aimed at comparing the performance of routine RDTs and smear microscopy with a simple molecular-based colorimetric loop-mediated isothermal amplification (LAMP) at two different levels of the health care system in a malaria-endemic area of western Kenya. Patients presenting with clinical symptoms of malaria at Rota Dispensary (level II) and Siaya County Referral Hospital (level IV) were enrolled into the study after obtaining written informed consent. Capillary blood was collected to test for malaria by RDT and microscopy at the dispensary and county hospital, and for preparation of blood smears and dried blood spots (DBS) for expert microscopy and quantitative real-time polymerase chain reaction (qPCR). Results of the routine diagnostic tests were compared with those of malachite green loop-mediated isothermal amplification (MG-LAMP) performed at the two facilities. The feasibility of using MG-LAMP for routine diagnosis was assessed using structured-questionnaire administered to health workers and study participants. A total of 264 participants were enrolled into the study. At the dispensary level, the positivity rate by RDT, expert microscopy, MG-LAMP and RT-PCR was 37%, 30%, 44% and 42%, respectively, while the county referral rates were 42%, 43%, 57% and 43%, respectively. Using qPCR as the reference test, the sensitivity of RDT and MG-LAMP was 78.1% (CI 67.5–86.4) and 82.9% (CI 73.0–90.3) at Rota dispensary. At Siaya hospital the sensitivity of routine microscopy and MG-LAMP was 83.3% (CI 65.3–94.4) and 93.3% (CI 77.9–99.2), respectively. Compared to MG-LAMP, there were 14 false positives and 29 false negatives by RDT at Rota dispensary and 3 false positives and 13 false negatives by routine microscopy at Siaya Hospital. Majority (64%) of study participants in both facilities disagreed that MG-LAMP can be used for malaria diagnosis in a health facility. MG-LAMP had a higher sensitivity than RDTs and microscopy in the detection of *Plasmodium* at the public health facilities and might be a useful quality control tool in resource-limited settings. The MG-LAMP assay had a shorter turn-around time compared to qPCR and can be easily used in a health facility.

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria remains a major public health problem and an impediment to social and economic development, particularly in sub-Saharan Africa. In 2017, of the estimated 219 million cases and 445,000 deaths attributed to malaria worldwide, approximately 90% of cases and deaths were in sub-Saharan Africa (WHO, 2018). Between 2000-2015, there was a significant reduction in the global malaria burden, with decline in incidence by 37% and mortality by 60% (WHO, 2018). However, over the last two years the rate of decline has stalled and even reversed in some regions. This has been attributed to several interconnected challenges including the fact that most people who are infected are not properly diagnosed and therefore do not receive appropriate treatment (WHO, 2016).

Accurate parasitological diagnosis of a malaria case using either quality-assured microscopy or Rapid Diagnostic Tests (RDTs) and prompt treatment with effective artemisinin-based combination therapies (ACTs) remains a key strategy in malaria case management and has played an important role in the reduction of the global malaria burden over the last two decades (WHO, 2017). In addition, pillar 1 of the Global Technical Strategy for Malaria recommends the universal access to malaria prevention, diagnosis and treatment for effective disease management and for surveillance (WHO, 2015). Concerted efforts by national malaria programmes to improve malaria parasitological diagnosis and strategies such as test, treat and track (T3) launched by World Health Organization (WHO) in 2012 have led to a significant increase in the number of health facilities in sub-Saharan Africa with capacity for microscopy or RDT (Bastiaens, 2014; Kachur, 2016).

However, this is not supported by robust in-country quality assurance/quality control (QA/QC) programs. This is evident from the findings of several studies that have evaluated the quality of diagnostic capacities in endemic areas, which have reported gaps in malaria microscopy ranging from shortage of trained personnel (Sori *et al*., 2018), lack of well-maintained microscopes and quality reagents (Wanja *et al*., 2017),

high workloads (Odhiambo *et al*., 2017) and poor performance in species identification and reporting (Jemere *et al*., 2018). Similarly, although RDTs are recommended for malaria diagnosis in health facilities where microscopy is not available and at the community level, their performance depends on several factors including; parasite density, patient antimalarial treatment history, *pfhrp2/3* deletions, storage conditions and operator proficiency (WHO, 2011). Without robust QA/QC systems, these factors could affect the diagnostic performance of RDTs or smear microscopy resulting in erroneous results, poor management of patients, irrational use of antimalarial drugs and inaccurate surveillance data. Whereas, nucleic acid amplification tests (NAATs) are several orders of magnitude more sensitive than RDTs and microscopy, WHO recommends that NAATs be considered only for epidemiological research and survey mapping of sub-microscopic infections (WHO, 2014). Nucleic acid amplification tests such as RT-PCR, quantitative nucleic acid sequence-based amplification (QT-NASBA) could be used as reference tests for QA/QC programmes, however, they are prohibitively expensive due to the high cost of equipment required, expensive reagents and the need for highly skilled laboratory personnel (Berzosa *et al*., 2018). Availability of other NAATs such as the loop-mediated isothermal amplification (LAMP) that do not require thermal cyclers or highly skilled laboratory personnel (Lau *et al*., 2016) could be an inexpensive reference test that can be used in a QA/QC programme in resource-limited settings.

In Kenya, parasitological diagnosis of malaria using microscopy or RDTs is recommended for all patients with suspected malaria (MoH, 2016). Since microscopy is only available at level III (health center) to level VI facilities (referral hospital), RDTs are used at level I (Community Health Workers) and level II facilities (dispensaries) or when microscopy is not available at other levels such as when there is power outage or stock out of reagents for microcopy. As in other endemic countries, the Kenya National Malaria Control Programme has developed malaria diagnosis and QA/QC guidelines and manuals (MoH, 2016). However, implementation has remained a challenge and this is likely to have an impact on patient management and tracking the malaria burden at the different levels of the health care system. Additionally, there

is limited information on how many patients are missed by routinely performed RDTs and smear microscopy at different levels of the healthcare system.

The main objective of the study was to compare the performance of routine RDTs and microscopy against an easy-to-use and highly sensitive molecular diagnostic assay, malachite green loop-mediated isothermal amplification (MG-LAMP) at two government health facilities representing different levels of healthcare delivery in Kenya. Although the use of LAMP has been extensively evaluated for malaria diagnosis in areas of low malaria transmission and elimination settings, there is limited information on their use to support a QA/QC system in resource-limited settings.

1.2 Statement of the Problem

Diagnosis of malaria by microscopy or RDTs before treatment is recommended by WHO in all patients suspected of having malaria. However, these diagnostic methods have limitations which could lead to inaccurate diagnosis due to poor sensitivity and specificity, especially in detecting low malaria parasite densities, resulting in inappropriate use of antimalarial drugs. The staining process and result interpretation during microscopy are labor intensive, time consuming, and require expertise and trained healthcare personnel, particularly for identifying the species accurately at low malaria parasitemia or in mixed infections. The current RDTs target the histidine rich protein-2 (HRP-2) gene, the target antigen in >90% RDTs, expressed only by *Plasmodium falciparum* hence cannot differentiate other malaria species. The HRP-2 can persist in the blood for several days even after the parasites are cleared, therefore the RDTs cannot accurately differentiate whether it is a current or recently treated infection. Furthermore, the recent discovery that up to 40% of *P. falciparum* parasites in parts of South America have the ability to delete the HRP-2 gene, has raised concerns about the use of RDTs in some settings. Molecular tests such as polymerase chain reaction are more sensitive but they require sophisticated laboratory infrastructure, making their implementation into malaria endemic areas expensive and challenging. The malachite green loop-mediated isothermal amplification is an isothermal nucleic acid amplification assay which is sensitive than the routine malaria tests and simpler and inexpensive than PCR, thus making it capable of filling this gap.

1.3 Justification

The impact of any malaria control program is determined by the reduction in malaria cases and transmission. In order for this to be determined, it is important that all existing malaria cases, including sub-microscopic or sub-RDT infections, are detected. The currently used detection methods; microscopy and RDT are not capable of detecting low-density malaria cases that molecular tools are capable of detecting. The MG-LAMP assay has a great potential to extend the reach of molecular tools which are highly sensitive and specific not only to settings where they are needed, but also where the sophisticated infrastructural requirements of conventional PCR-based diagnostic methods are not available. However, it is crucial that these novel techniques be evaluated in malaria endemic regions where their validity as a sensitive field-usable molecular diagnostic tool for malaria can be established. The results from this study will provide important information on the feasibility of using the MG-LAMP assay in a malaria endemic field setting allowing health care practioners to make an informed decision as to its applicability. The people of sub-Saharan Africa are known to be poor, thus, inexpensive and affordable diagnostic tools are required. Therefore, the study also assessed the applicability of MG-LAMP assay as an inexpensive alternative to PCR which can be used in QA/QC systems at health facilities in malaria endemic resource-limited areas.

1.4 Research Questions

1. What is the diagnostic performance of malachite green loop-mediated isothermal amplification (MG-LAMP) assay in detecting *Plasmodium* species at the two different health facilities in a malaria endemic area of western Kenya?
2. Can the malachite green loop-mediated isothermal amplification (MG-LAMP) assay be used at local health facilities in a malaria endemic area of western Kenya?

1.5 Objectives

1.5.1 General Objective

To evaluate the colorimetric malachite green loop-mediated isothermal amplification (MG-LAMP) assay for the detection of *Plasmodium* species at two different health facilities in a malaria endemic area of western Kenya.

1.5.2 Specific Objectives

1. To determine the diagnostic performance of the colorimetric malachite green loop-mediated isothermal amplification (MG-LAMP) assay for the detection of *Plasmodium* species at two different health facilities in a malaria endemic area of western Kenya.

2. To determine the feasibility of using the colorimetric malachite green loop-mediated isothermal amplification (MG-LAMP) assay for the detection of *Plasmodium* species at two different health facilities in a malaria endemic area of western Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Malaria, sometimes nicknamed "King of Diseases", is caused by protozoa parasite of the genus *Plasmodium* (Hoffman & Stephen, 1996). The *Plasmodium* parasites are spread through the bites of infected female *Anopheles* mosquitoes, which are the malaria vectors (WHO, 2017). There are 5 *Plasmodium* species that cause malaria in humans; *Plasmodium falciparum, Plasmodium malariae, Plasmodium vivax, Plasmodium ovale* and *Plasmodium knowlesi* (WHO, 2017). *P. falciparum* causes the most serious and fatal type of malaria, while the other species, *P. vivax, P. ovale, P. malariae,* and sometimes *P. knowlesi* can cause acute, severe illness but mortality rates are low (Tangpukdee *et al*., 2009). According to the National Malaria Control Programme (NMCP), *P. falciparum* accounts for 98% of all malaria infections in Kenya (National Malaria Control Program, 2016). The principal vectors of malaria parasites in Kenya are members of the *Anopheles gambiae complex* and *An. funestus*. The *An. gambiae complex* species found in Kenya are; *An. gambiae s.s., An. arabiensis* that is usually predominant during and after the rainy seasons, *An merus*, which is mainly restricted to the coastal strip and *An funestus* exist in low densities throughout the year (National Malaria Control Program, 2016).

2.2 Malaria Burden

Malaria is the most important infectious disease in the tropical and subtropical regions. It continues to be a major global health problem, with over 40% of the world's population exposed to varying degrees of malaria risk in some 100 countries (Tangpukdee *et al*., 2009). According to WHO According to the WHO, in 2017, an estimated 219 million cases of malaria and 435,000 deaths occurred worldwide compared with 217 million cases in 2016 (WHO, 2018). The 2009 WHO data indicate that there were 225 million cases of malaria, resulting in 781,000 deaths, followed by marked improvement in 2010, when the number of cases declined to 216 million and the number of deaths to 655,000 (Wilson, 2013). Between the year 2010 and 2015, the incidence of malaria among the risk populations fell by 21% globally, while during the same period, the rate of malaria mortality among populations at risk decreased by

29%. An estimate of 6.8 million malaria deaths have been averted globally since 2001 (WHO, 2017). In 2015, the WHO African region accounted for 90% of malaria cases and 92% of malaria deaths. Some thirteen countries, mainly in sub-Saharan Africa, account for 76% of malaria cases and 75% deaths globally (WHO, 2017). In areas with high malaria transmission, children under five-years of age are more susceptible to malaria infection, illness and death and more than two thirds (70%) of all malaria deaths occur in this age group. Between 2010 and 2015, the malaria death rate of children under five-years fell by 29% globally (WHO, 2017). However, malaria still remains a major killer of children under five years old, taking the life of a child every two minutes.

In Kenya, malaria is the one of the leading causes of morbidity and mortality. Over 25 million Kenyans were at risk of malaria (National Malaria Control Program, 2016). It is the biggest childhood killer with an estimated 34,000 children under five years dying each year and it contributes to 30% to 50% of outpatient visits at health facilities and 19% of all hospital admissions (National Malaria Control Program, 2016). Malaria represents a significant economic burden with an estimated 170,000 million working days a year lost due to malaria illness (National Malaria Control Program, 2016). However, there has been a steady decline of malaria cases in the last five years. In Kenya, malaria distribution is not uniform due to geographical differences in altitude, rainfall and humidity (National Malaria Control Program, 2016). There are four malaria epidemiological zones in Kenya (PMI, 2014) and they include:

a) **Endemic zones** comprise of areas with altitudes ranging from 0 to 1,300 meters and include the Lake Victoria in western Kenya and the Coastal regions of Kenya. The prevalence of *P. falciparum* in these areas falls between 20% to 40% and these areas also have a high annual entomological inoculation rate.
b) **Highland epidemic-prone areas**: In these areas, malaria transmission is seasonal with considerable year-to-year variation. These areas include the western highlands of Kenya. The prevalence of *P. falciparum* malaria ranges from 1% to 5%, but can be higher as 10% to 20%.
c) **Seasonal malaria transmission areas**: These areas include the arid and semiarid areas of northern and southeastern parts of the Kenya. These areas

experience short periods of intense malaria transmission during the rainy seasons. The malaria prevalence in these areas is less than 5%.

d) **Low malaria risk areas**: The central highlands of Kenya including Nairobi lie in this zone. In these areas, there is little to no malaria transmission.

2.3 Life-Cycle of the Malaria Parasite

The life cycle of malaria parasites is essentially the same (Figure 2.1). It comprises of an exogenous sexual phase, sporogony, with multiplication in the gut of female *Anopheles* mosquitoes and an endogenous asexual phase, schizogony, which takes place in the vertebrate host (man) (Cuomo *et al*., 2009). The latter phase includes the development of the parasites in the erythrocytes (erythrocytic schizogony) and the phase which takes place in the parenchyma cells in the liver (pre-erythrocytic schizogony) (Cuomo *et al*., 2009). The life cycle of a malaria parasite involves two hosts (CDC, 2009). When a female *Anopheles* mosquito takes a blood meal from an infected person, it ingests blood which contains the mature male and female gametocytes, which then undergo a series of developmental cycles in the gut of the mosquito (Paniker, 2013). Ex-flagellation occurs, which results in the production of a number of male and female gametes, then fertilization occurs producing a zygote which matures to an ookinete. The ookinete penetrates the gut wall of the mosquito where it grows into an oocyst and which further matures to become a motile sporozoite (Paniker, 2013).

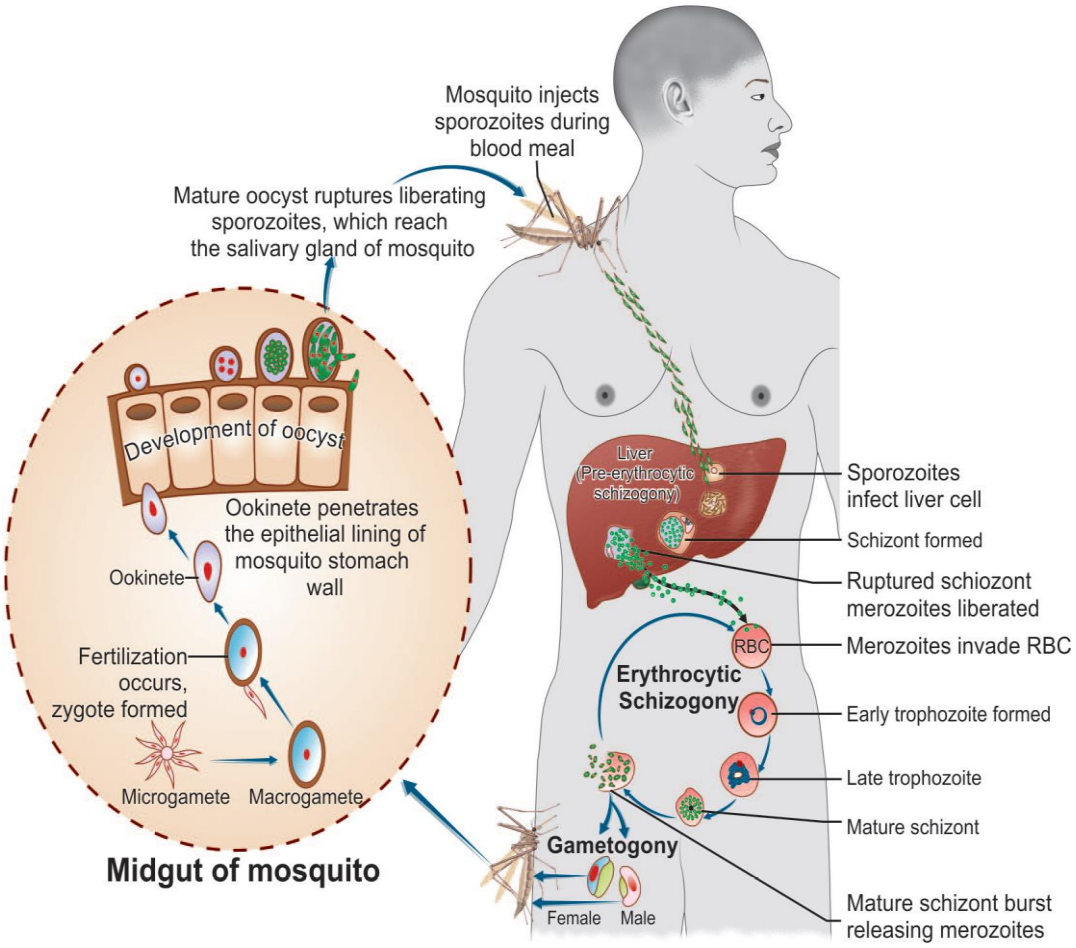

Figure 2.1: Life cycle of malaria parasite

Source: (Paniker, 2013)

The sporozoites then migrate from the body cavity of the mosquito to the salivary glands, which makes the mosquito to be infective. During a blood meal, the sporozoites enter into the blood stream of a host. Following the inoculation, the sporozoites leave the blood stream within 40 minutes and then enter the parenchymal cells of the liver cells (hepatocytes) (Satoskar *et al*., 2009). In all the four species, the asexual development occurs in the hepatocytes, a process referred to as pre-erythrocytic schizogony**,** which produces thousands of tiny merozoites which are released into the circulation after 14 to 16 days. However, in the case of *P. vivax* and *P. ovale*, some sporozoites differentiate into hypnozoites which remain dormant in hepatocytes for considerable amount of time (Paniker, 2013). When the hypnozoites are reactivated, they undergo asexual division to produce a clinical relapse. In *P. falciparum* and *P.*

malariae, the hypnozoites are not formed, therefore, the parasite develops directly into pre-erythrocytic schizonts (Paniker, 2013). When the merozoites are in the circulation, they invade the erythrocytes and develop into trophozoites. During the course of their development, they are able to absorb the hemoglobin of the red blood cells and leave a by-product of digestion called hemozoin in form of a pigment, which is a combination of hematin and protein (Paniker, 2013). This iron-containing pigment is observed in the body of the parasites as dark granules, which are more obvious in the later stages of development (Cuomo *et al*., 2009). After a while, the trophozoites undergo asexual division, i.e. erythrocytic schizogony. As the mature trophozoites start to divide in the erythrocytes, separate merozoites are formed which result in a schizont (Satoskar *et al*., 2009). When fully developed, the schizont ruptures the erythrocyte, releasing the merozoites into the circulation. These merozoites then infect new erythrocytes and the asexual reproduction cycle in the blood proceeds (Garcia, 2007). Some of the merozoites which penetrate the erythrocytes do not form trophozoites, but they develop into gametocytes and this process takes place in deep tissue capillaries. This erythrocytic cycle of schizogony is repeated during the course of the malaria infection, leading to a progressive increase of parasitemia (Cuomo *et al*., 2009).

2.4 Clinical Manifestations of Malaria

The clinical symptoms of malaria result from the schizont rupture and destruction of erythrocytes. Malaria disease can either have a gradual or a fulminant course with non-specific symptoms (Prabhu *et al*., 2003). The clinical manifestations, severity and the course of a clinical attack depends on the species and the strain of the infecting *Plasmodium* parasite, as well as the age, genetic constitution, immune status, malaria specific immunity and nutritional status of the child, mode of transmission, whether the individual was on prophylaxis or had previous exposure to antimalarial drugs, as this may present with only minimal symptoms or signs (Schumacher & Spinelli, 2012). The most characteristic malaria symptom is fever. Other common symptoms which have been seen in malaria include chills, headache, myalgias, nausea, and vomiting (Garcia, 2007). Diarrhea, abdominal pain, and cough are occasionally seen (Paniker, 2013). As the malaria disease progresses, some patients develop malaria paroxysms

with bouts of illness alternating with symptom-free periods (Garcia, 2007). The first paroxysm is a cold stage which lasts 15 to 60 minutes, and characterized by shivering and a feeling of cold. The next is the hot stage which lasts 2 to 6 hours, in which there is fever, which sometimes reaches 41°C, flushed, dry skin, and often headache, nausea, and vomiting. The last stage is the sweating stage, lasting 2 to 4 hours during which the fever drops rapidly and the patient sweats (Paniker, 2013). These malaria paroxysms result from the lysis of parasitized erythrocytes and release of merozoites into the circulation as the asexual reproduction comes to an end (Schumacher & Spinelli, 2012). The following are the major complications of severe malaria: cerebral malaria, pulmonary edema, acute renal failure, severe anemia, and/or bleeding. Acidosis and hypoglycemia are the most common metabolic complications. Any of these complications can develop rapidly and lead to death within hours or days if the infection is not treated (Prabhu *et al*., 2003).

2.5 Clinical and Laboratory Diagnostic Methods of Malaria Infection

The World Health Organization recommends prompt and accurate parasitological confirmation of malaria diagnosis by optic microscopy or rapid diagnostic tests based on lateral flow immunochromatography as part of an effective disease management. Delays in diagnosis are associated with an increased risk of severe malaria, requirement for intensive care and death (Schumacher & Spinelli, 2012). Clinical diagnosis is imprecise but it still remains the basis of therapeutic care for the majority of febrile malaria patients in endemic areas, where laboratory support is often unavailable (Wernsdorfer *et al*., 2007).

2.5.1 Clinical Diagnosis of Malaria

This is the cheapest, most commonly used method of malaria diagnosis and is the basis for self-treatment (Wernsdorfer *et al*., 2007). The earliest symptoms of malaria are non-specific and are variable, and they include fever, headache, weakness, myalgia, chills, dizziness, abdominal pain, diarrhea, nausea, vomiting, anorexia, and pruritus (Tangpukdee *et al*., 2009). However, the overlapping of malaria symptoms with other tropical diseases impairs its specificity, therefore, encouraging the indiscriminate use of anti-malarial for managing febrile conditions in malaria endemic areas (Wernsdorfer *et al*., 2007). The Integrated Management of Children Illness (IMCI) has

provided clinical algorithms which can be used in managing and diagnosing common childhood illnesses by healthcare providers who have minimal training in the developing countries (Tangpukdee *et al*., 2009). A study conducted in Kenya (Mwangi *et al*., 2005) showed that clinical algorithms therefore appeared to have little utility in malaria diagnosis, performing even worse in the older age groups, where avoiding unnecessary use of anti-malarials would make more drugs available to the really needy population of children under 5-years of age. Other studies conducted in Mali, (Dicko *et al*., 2005) and Tanzania, (Drakeley *et al*., 2006), have shown a wide range of percentages of malaria over-diagnosis and its associated potential for economic loss.

2.5.2 Laboratory Diagnosis of Malaria

There are different techniques which can be used to diagnose malaria in a laboratory, for example, conventional malaria microscopic diagnosis which involves preparation of thick and thin blood smears (Ngasala *et al*., 2008), rapid diagnostic assays such as OptiMAL (Zerpa *et al*., 2008; Tagbor *et al*., 2008) and molecular diagnostic methods, such as polymerase chain reaction (Vo *et al*., 2006). In malaria endemic countries, such as Kenya, the current routine malaria diagnostic tools are microscopy and RDTs (NMCP, 2016).

2.5.2.1 Microscopy

The current accepted routine diagnostic technique for malaria infection is the microscopic examination of stained blood smears, stained with either Giemsa or Wright's stain and observed under the oil immersion lens (WHO, 2014). It is the gold standard for detecting malaria parasitemia (Wilson, 2013). The microscopy method has changed very little since Laverran's original discovery of the malaria parasite and improvements in staining techniques by Romanowsky in the late 1800s (Tangpukdee *et al*., 2009). Microscopy is based on examination of both thick and thin blood smears made from the same sample of peripheral blood (Wilson, 2013). The thick blood smear is used for the detection of malaria parasites, while the thin blood smear is for identification of parasite species (Bailey *et al*., 2013). The thick blood smear consists of many layers of red and white blood cells (WHO, 2010). During staining, the hemoglobin in the red cells de-hemoglobinizes, so that large amounts of blood can be examined quickly and easily. Malaria parasites, when present, are more concentrated

than in a thin film and are easier to see and identify (WHO, 2010). The thin blood smear is used to search for parasites only in exceptional situations. A well-prepared thin blood film consists of a single layer of red and white blood cells spread over less than half the slide (WHO, 2010).

Microscopic diagnosis of malaria has many advantages. For example, it has low direct costs if the infrastructure to maintain the service is already available, it is sensitive if the quality of microscopy is high, it can differentiate between malaria species, it can determine parasite densities, it can also be used to diagnose other diseases (WHO, 2009). Although microscopy has these advantages, it has been difficult to maintain good quality microscopy especially at the periphery of the health services. where most patients are being treated (WHO, 2009). The current limitations of malaria microscopy are well recognized and documented and they include (WHO, 2009): lack of political commitment to support the development and expansion of laboratory services, lack of funds to support the integration of malaria diagnosis into the general laboratory services, poor quality of microscopy particularly at the peripheral level, difficulties in maintaining microscopy facilities in good order, logistic problems and high costs of maintaining adequate supplies and equipment, lack of adequate training and retraining of laboratory staff, delays in providing results to clinical staff, lack of quality assurance and supervision of laboratory services; and inability to cope with the workload of traditional systems for cross-checking of routinely taken malaria slides, often due to inadequate human and financial resources.

These limitations can only be overcome by new health policies that acknowledge the importance of strengthening laboratory services, the need for adequate funding and the implementation of a quality assurance system (WHO, 2009).

2.5.2.2 Malaria Rapid Diagnostic Test (RDT)

A malaria rapid diagnostic test is a device that detects malaria antigen in a small amount of blood by immunochromatographic assay with monoclonal antibodies directed against the target parasite antigen and impregnated on a test strip (Wernsdorfer *et al*., 2007). Immunochromatography relies on the migration of a liquid across the surface of a nitrocellulose membrane (Moody, 2002). These tests rely on

the capture of antigen on the malaria parasite from the peripheral blood by use of monoclonal antibodies that are prepared against a malaria antigen target and then conjugated to either a liposome containing selenium dye or gold particles in a mobile phase. A second or third capture monoclonal antibody is applied to a strip of nitrocellulose that acts as the immobile phase (Moody, 2002). Characteristics required of a rapid malaria diagnostic test vary depending on regional malaria epidemiology and the goals of the malaria control program (Miller *et al*., 2008). The two most important characteristics needed for a diagnostic test in order to reduce mortality from malaria in sub-Saharan Africa are high sensitivity for detecting *P. falciparum* and rapid availability of test results (Miller *et al*., 2008). Other characteristics of malaria rapid tests include: the diagnostic test should use simple technology, should be readily learned by users, should have results that are easy to interpret and reproducible, should not require electricity to run the assay, and should not require refrigerated storage (Wilson, 2013). Malaria antigens currently used as diagnostic targets are either specific to a *Plasmodium* species or conserved across the human malarias. *P. falciparum*-specific monoclonal antibodies have been developed for histidine-rich protein 2 (HRP-2) and *P. falciparum* lactate dehydrogenase (pLDH) (Miller *et al*., 2008). Currently, there are 86 malaria RDTs available from 28 different manufacturers (Tangpukdee *et al*., 2009). Malaria RDTs have provided an opportunity to extend the benefits of parasite-based diagnosis of malaria beyond the confinement of light microscopy, with significant advantages in the management of febrile illnesses in remote malaria endemic areas (Tangpukdee *et al*., 2009).

Although HRP-2-based malaria RDTs permit rapid diagnosis of falciparum malaria, their clinical usefulness in the diagnosis of the other *Plasmodium* species and for monitoring of therapeutic response is limited (Moody, 2002). The HPR-2 is only expressed by *P. falciparum*, which will give negative results with samples containing only *P. vivax, P. ovale*, or *P. malariae*. Many cases of non-*falciparum* malaria may, therefore, be misdiagnosed as malaria negative cases (Moody, 2002). Other recognized disadvantages of RDTs include: persisting HRP-2 antigenaemia can give a positive test when no viable parasites are present; in the case of *P. falciparum* or *P. vivax* infection, the species cannot be determined; quantification of malaria parasite is not

possible; the operator misunderstanding or misinterpretation of test line patterns may lead to apparent discrepancy between RDT and blood film results; and a prozone effect may occur with HRP-2 based RDTs (Bailey *et al*., 2013).

2.6 Molecular Diagnosis of Malaria

Molecular methods based on DNA amplification have been applied to malaria diagnosis since the late 1980s (Rougemont *et al*., 2004), with their value lying in their high sensitivity, detecting ≤5 parasites/µl. Amplification of DNA has provided the opportunity to devise highly sensitive methods of malaria parasite detection, and the specificity inherent to these methods allows the unequivocal identification of the parasite species (Snounou, 2002).

2.6.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) based methods have been in use since the early 1990's for the detection of *Plasmodium* parasites (Coleman *et al*., 2006). This technique has continued to be extensively used as a confirmatory test for malaria infections, to follow-up therapeutic response, and to identify drug resistance (Tangpukdee *et al*., 2009). The efficiency of the assay markedly improved when nested-PCR strategy is adopted (Snounou, 2002), in which two rounds of amplification are carried out, with the product of the first reaction serving as the template for a second reaction where the oligonucleotide primers used hybridize to sequences contained within that product (Snounou, 2002). With nested or semi-nested PCR methods targeting the small-subunit 18S rRNA gene, all four species can be identified (Rougemont *et al*., 2004). A study conducted by (Coleman *et al*., 2006) comparing PCR and malaria microscopy showed increased sensitivity in comparison to microscopy for the diagnosis of mixed infection.

Many studies have shown that, although PCR is sensitive and specific for the malaria diagnosis, there are limitations that affect the accuracy of the method (Coleman *et al*., 2006). These limitations include; selecting appropriate primers, methods used for collection and storage of blood samples, and extraction methods used can all affect the performance of PCR (Coleman *et al*., 2006). Furthermore, the WHO recommends that NAATs be considered only for epidemiological research and survey mapping of sub-

microscopic infections (WHO, 2014). A report by (Jelinek *et al*., 1996), demonstrated that the sensitivity of PCR was linked to parasite density as in the case of microscopy. The study found that the PCR's sensitivity was affected by both the parasite density and by the geographic differences in parasite populations. Conventional PCR assays are technically demanding and time-consuming. Moreover, they are prone to carry over contamination during the manipulation of post-amplification products, a problem already observed in the unique study testing the use of PCR as a routine method for malaria diagnosis (Rougemont *et al*., 2004), hence it is not routinely implemented in developing countries where malaria is endemic because of the testing complexity and due to the lack of resources to perform the tests adequately and routinely (Tangpukdee *et al*., 2009). However, as progress is made towards better malaria control and eventual goal of elimination, more sensitive diagnostic tools will be required in order to detect asymptomatic low level parasitemia (Lucchi *et al*., 2010). Therefore, further efforts are needed to develop next generation molecular tools for field use with a goal that such tools can complement, or in some situations, replace the existing molecular methods for malaria diagnosis and operational programs such as monitoring and evaluation of control and elimination programs (Lucchi *et al*., 2010).

2.6.2 Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is an isothermal DNA amplification method that was developed by laboratory scientists at Eiken Chemical Company of Japan (F.I.N.D, 2012), in 1998. According to the Foundation for Innovative New Diagnostics (F.I.N.D) the LAMP technology amplifies previously determined genes and can be used to detect any pathogen (F.I.N.D, 2012). It might be considered as an alternative to PCR for the detection of nucleic-acid sequences. DNA amplification is accomplished with the use of DNA polymerase with strand-displacing activity, *Bacillus stearothermophilus*, (Drapala & Kordalewska, 2013). Both methods amplify and detect DNA, but unlike traditional PCR, LAMP does not require thermocycler or gel imaging system (F.I.N.D, 2012). Amplification and detection of the target nucleic-acid sequence is essentially completed in a single step, by incubating the mixture of sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (Drapala & Kordalewska, 2013). LAMP provides high

efficiency, with DNA being amplified 10^9-10^{10} times in 15 - 60 minutes (F.I.N.D, 2012). Therefore, LAMP can provide faster results than traditional PCR, and can be performed in basic laboratories without the need for specialized infrastructure (Lucchi *et al*., 2016).

The LAMP technology is characterized by: the use of a single polymerase enzyme to catalyze DNA amplification under isothermal conditions, very high specificity that results from the use of six primers recognizing eight distinct regions on the target DNA, and high amplification efficiency capable of producing high concentrations of amplified product in a short time, allowing for visual or automated detection of results (F.I.N.D, 2012). Another advantage of LAMP reaction is its robustness and tolerability to common PCR inhibitors which allows use of a simplified sample preparation that just requires boiling and centrifugation, or use of 'PURE' device that rapidly removes impurities from the DNA sample (F.I.N.D, 2012). The whole LAMP reaction consists of two steps: non-cyclic and cyclic (Drapala & Kordalewska, 2013), which begins with short single stranded molecules called oligonucleotides or primers (F.I.N.D, 2012). These primers are designed to bind to the target DNA sequence, whereby, if the parasite DNA is present, one of the specially designed LAMP primers can anneals to the complementary DNA from the parasite (Drapala & Kordalewska, 2013). This occurs because the DNA is in dynamic equilibrium when it reaches the reaction temperature. Primer binding initiates the process of DNA synthesis, whereby the *Bst* DNA polymerase enzyme generates new DNA that matches the parasite DNA (Drapala & Kordalewska, 2013). As the DNA synthesis progresses, some of the new DNA folds back on itself to form a "stem-loop" structure that looks like a dumbbell (F.I.N.D, 2012). This structure is the starting point for the amplification cycle of LAMP. The loops on the dumbbell structure now act as additional primers for on-going DNA synthesis (F.I.N.D, 2012). As more loops are created, there are more starting points for DNA synthesis as the LAMP reaction continues (Drapala & Kordalewska, 2013).

The automated detection of amplified products is based on turbidimetric measurement of magnesium pyrophosphate while visual detection under ultraviolet light is based on the presence of calcein (Lucchi *et al*., 2016). Before DNA amplification, calcein

contained in the reagent is in its quenched state as it is bound to manganese ions, but as start of DNA amplification starts, pyrophosphate ions bind to manganese ions and calcein is released producing fluorescence (Drapala & Kordalewska, 2013). Different colorimetric approaches for LAMP product readout have been investigated with some success. Recently, a malachite green-based LAMP assay was used for the detection of malaria species (Lucchi *et al*., 2016).

2.7 Global Malaria Control Strategies

In 2000, it was estimated that 86% of malaria deaths occurred in children aged under 5 years. Malaria accounted for 12% of all deaths in children under 5-years of age globally and 22% in sub-Saharan Africa (WHO & UNICEF, 2015). Due to the devastation caused by malaria, combating the disease, along with other infectious diseases such as HIV/AIDS, was identified as a priority at the 2000 United Nations General Assembly (United Nations, 2000), and was designated as Goal 6 of the eight Millennium Development Goals (MDGs). Despite the unpromising circumstances in 2000 for achieving the MDG 6 target, from 2000 to 2015 saw impressive reductions in malaria, with incidence rates falling by 37% globally and death rates by 60% (WHO & UNICEF, 2015).

On the path towards malaria elimination, countries, subnational areas and communities are situated at different points, and their rate of progress differs depending on the investment level, biological determinants, environmental factors, strength of health systems as well as social, demographic, political, and economic factors (WHO, 2016). There is an urgent need to adopt and expand the implementation of WHO recommended strategies to increase the effectiveness of responses and to bring an end to preventable malaria deaths. For this to be achieved, new and improved tools and approaches are available (WHO, 2016). These strategies are built on three pillars with two supporting elements (WHO, 2016), that guide global efforts to in malaria elimination:

Pillar 1. Ensuring universal access to services relating to malaria prevention, diagnosis and treatment. The WHO recommends that quality-assured vector control, chemoprevention, diagnostic testing and treatment, can drastically reduce morbidity

and mortality. In areas where there is moderate-to-high transmission, ensuring universal access of populations at risk to malaria interventions should be a principal objective of national malaria control programs. The WHO recommends the implementation of two sets of interventions: prevention strategies based on vector control, and, in some settings and some population groups, administration of chemo-prevention, and universal diagnosis and prompt effective treatment of malaria in public and private health facilities and at community level. This pillar has been enhanced by the recent advances in the development of malaria vaccines.

There are two pre-erythrocytic malaria vaccines which are undergoing clinical trials, that is, RTS,S/AS01 (Mosquirix) malaria vaccine, which was developed by GlaxoSmithKline Biologicals (GSK, UK) and the PATH Malaria Vaccine Initiative (MVI), and PfSPZ malaria vaccine candidate which was developed by Sanaria Inc (USA) (Jadhav *et al*., 2012; MaHTAS, 2017). The RTS,S/AS01 is the only malaria vaccine candidate which has reached phase 3 trials while the PfSPZ malaria vaccine is at phase 2 clinical trials (MaHTAS, 2017). These malaria vaccines will play a key role in MDG 6, in the prevention and control of malaria as well as enhancing malaria elimination and eradication strategies.

Pillar 2. Accelerating efforts towards eliminating and attainment of malaria-free status. It is necessary for countries to intensify efforts to reduce onward transmission of new infections in defined geographical areas, particularly in settings where there is low transmission. Attaining this objective involves targeting both the parasites and the vectors in a well-defined transmission focus, and with the guidance of active case detection and case investigations as part of a malaria surveillance and response program. Developing and adopting innovative solutions is essential to respond to the spread of insecticide resistance and residual transmission, and also to target the hypnozoite reservoirs of *P. vivax*.

Pillar 3. Transforming malaria surveillance programs in to core interventions. In countries where malaria is endemic and in those which are susceptible to the re-establishment of malaria, should have an effective health management and information system in place for helping national malaria control programs to direct resources to the

most affected populations, identify gaps in the program coverage, detect outbreaks, and to assess the impact of interventions in order to guide changes in program orientation. In areas of low malaria transmission, surveillance should trigger a locally-tailored response to every detected infection, detecting gaps in program coverage, declines in effectiveness of diagnostic tools, or the occurrence of outbreaks.

There are two elements which support the three pillars (WHO, 2016):

Innovation harnessing and expanding research on malaria. To support these three pillars, malaria endemic countries and the global malaria community should harness innovation and increasingly engage in basic, clinical and implementation of malaria research. Successful innovation in product development and service delivery will contribute to accelerating progress. Basic research is important for a better understanding of the parasites and the vectors, and helps in the development of more effective diagnostics and medicines, improved and innovative vector control methods, and vaccines.

Strengthen the enabling environment. There is need for multi-sectoral collaboration through strong political commitment, and robust financing, to make proper progress in malaria control and elimination. For optimum national malaria responses, there is need for an overall strengthening of health systems and improvement in the enabling environment. Strong health systems, both public and private, are essential for reducing the disease burden and transmission of malaria parasites, and enable the adoption and the introduction of new diagnostic tools and control strategies within the shortest possible time frame. Additionally, the expansion of malaria interventions should be used as an entry point for strengthening health systems, including maternal and child health programs and laboratory services, and also to build a stronger system for health information and for disease and entomological surveillance. Finally, empowering communities, capacity building and supportive supervision for a strong health workforce and regulatory frameworks are important for ensuring achievement of the vision, goal and milestones in the malaria control and elimination strategies.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was conducted at two health facilities (Rota Dispensary (level II) and Siaya County Referral Hospital (level IV)) which are located in an endemic area of western Kenya and serve mostly rural populations (Figure 3.1). Majority of the residents in this area belong to the Luo ethnic group and live-in scattered family compounds consisting of one or more houses surrounded by agricultural fields. The main occupation of the residents in this area includes subsistence farming, fishing and small-scale trading. The community prevalence of *Plasmodium falciparum* by slide microscopy is 28% in children aged <5 years, 42% in the 5- to 15-year-old and 18% in those aged >15 years (KEMRI-CDC unpublished data). Malaria is one of the main causes of hospital visits and admissions in this area (Kapesa *et al*., 2018). The primary malaria vectors are *Anopheles gambiae s.l.* and *An. funestus* and entomologic inoculation rates are <20 infective bites per person per year (Bayoh *et al*., 2014). Malaria transmission occurs year-round with two peak seasons from May-July and November-December coinciding with the end of the long and short rains respectively.

3.1.1 Organizational Structure and Design of Healthcare System in Kenya

The two health facilities are government-owned and are aligned with the country's health service delivery system; level-I (community), level-II (dispensaries/clinics), level-III (health centers/maternities/nursing homes), level-IV (sub-county hospitals), level-IV (county referral hospitals) and level-VI (regional and national hospitals). Dispensaries are headed by a nurse and provide promotive and preventive care. Malaria diagnosis at dispensary is primarily by RDT. County hospitals are headed by a medical officer and undertake mainly curative and rehabilitative services. Malaria diagnosis at this level is performed by laboratory technologists mainly by microscopy but RDTs can be used if microscopy is unavailable, for example when there is a prolonged power outage or stock outs of reagents for microscopy. Rota Dispensary records 20-30 patients per day mainly for outpatient consultations while complicated cases are referred to levels 3, 4 or 5 facilities. Siaya Hospital outpatient department

(OPD) records 40-60 patients per day with both minor and complicated ailments and has inpatient facilities. Any complicated cases are referred to level-6 facilities.

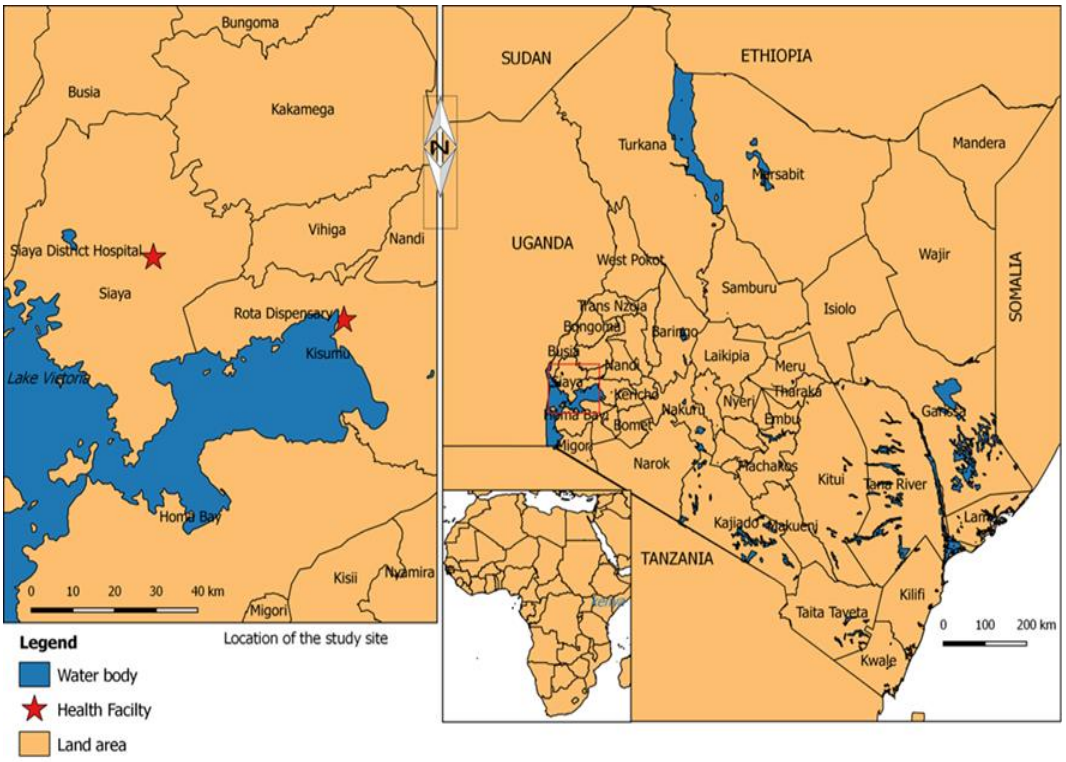

Figure 3.1: Map of Rota Dispensary and Siaya County Referral Hospital in western Kenya.

3.2 Study Design

The study was an experimental cross-sectional study which was carried out in a malaria endemic region of western Kenya.

3.3 Determination of Sample Size

The estimated sensitivity of LAMP from other published studies compared to PCR is shown to be above 90%. Sample size was determined using Tilaki's formulae (Hajian-Tilaki, 2014) as shown below. A pre-determined LAMP sensitivity was used.

$$N = \frac{Z_{\frac{\alpha}{2}}^2 Se(1 - Se)}{d^2 \times Prev}$$

where $Z_{\alpha/2}$=1.96

Se= pre-determined value of sensitivity that is ascertained by previous published data, 92% (Oriero *et al*., 2015), Prev= The prevalence of the disease based on PCR, 42% (Waitumbi *et al*., 2011), and d= Clinically acceptable width, 95% confidence interval.

$$N = \frac{1.96^2 \times 0.92 \times 0.08}{0.05^2 \times 0.42} = 269$$

3.4 Enrollment of Study Subjects

Study participants were recruited at the outpatient departments of the two health facilities if they presented with symptoms suggestive of malaria and were referred to the laboratory for malaria parasitological diagnosis. At the dispensary, RDT (SD Bioline Malaria Ag P.f/Pan 05FK60, Standard Diagnostics, Kyonggi, Republic of Korea) was used for diagnosis. Routine microscopy was used for diagnosis at the county referral hospital as per the national malaria diagnosis and treatment guidelines. Participants were enrolled into the study after obtaining written informed consent for participants aged over 18 years (Appendix I), parental/caregiver consent for those aged less than 18 years (Appendix III) or written assent for emancipated minors (Appendix II). Participants were excluded if they presented with severe disease or reported use of antimalarial drugs during the past four weeks. Participants found to be malaria positive were treated with artemether-lumefantrine (AL) as per the Kenya Ministry of Health national guidelines.

3.4.1 Inclusion Criteria

- Those who were 6 months old and above
- Those that provided written informed consent and assent
- For children <18 years of age, provide written caregiver consent (except for mature minors)

3.4.2 Exclusion Criteria

- Persons with severe disease or danger signs

3.5 Sample Collection

Approximately 300 µL of capillary blood was collected into ethylenediaminetetraacetic acid (EDTA) microtainers from enrolled participants. The whole blood was used for preparation of blood smears for expert microscopy, performing malaria RDT, performing MG-LAMP assay and preparation of dried blood

spots (DBS) on Whatman® 903 protein saver filter paper (GE Healthcare, USA) for qPCR. All samples were assigned a unique study identification number. Blood smears and DBSs were transported to Kenya Medical Research Institute/Centre for Global Health Research (KEMRI/CGHR) malaria laboratories, located about 6.1 kilometres (km) from Rota Dispensary and 56 km from Siaya County Referral Hospital, for storage and analysis.

3.6 Laboratory Procedures

3.6.1 Rapid Diagnostic Testing (RDTs) and Malaria Microscopy at the Two Health Facilities

Rapid diagnostic tests SD Bioline Malaria Ag P.f/Pan 05FK60 (Standard Diagnostics, Kyonggi, Republic of Korea) were performed at Rota dispensary using 5 µL of blood according to manufacturer's instructions. At the Siaya County Referral Hospital, malaria microscopy was performed by a laboratory technologist using the hospital standard operating procedure (SOP) and involved collection of finger prick blood, preparation of the thick and thin blood smears using 6 and 2 µL of blood respectively, staining using 10% Giemsa for 15 minutes and examination of slides under a microscope. A smear was considered negative if no parasites were detected in 100 high power microscopic fields.

3.6.2 Expert Microscopy at the KEMRI/CGHR Malaria Laboratories

For endpoint analysis, malaria microscopy was carried out according to WHO basic malaria microscopy guidelines, 2010 (WHO, 2010). Thick (for parasite density determination) and thin (for *Plasmodium* species identification) smears were prepared from 6 µL and 2 µL of whole blood sample respectively. The thick and thin smears were allowed to air dry and then stained using 3% Giemsa stain for 1 hour. The slides were read at 100x objective lens under oil immersion using a compound microscope for determination of both asexual and sexual stage of parasites. A blood smear was considered negative if 100 microscopic high-powered fields showed no parasites. If a blood smear was positive, malaria parasites were counted in 40 microscopic high-powered fields and parasite densities expressed per microliter (µL). All blood smears were examined independently by two expert microscopists blinded to each other's results. Where the two readings differed in results (one reader positive and the other

negative), parasite species, or if the higher count divided by the lower count was ≥2 (for high and medium parasitaemia) and ≥10 (for low parasitemia), smears were re-examined by a tie-breaker microscopist who was blinded to the results of the first two readers. All the microscopists were enrolled and had passed a quarterly external quality assurance program administered by the National Institute of Communicable Diseases (NICD), South Africa.

3.6.3 Malachite Green Loop-Mediated Isothermal Amplification (MG-LAMP) Assay

The MG-LAMP was performed at the health facilities by boil-and-spin method (Lucchi *et al*., 2016) using 50 µL of the collected blood. DNA was released from whole blood by boiling in a heat-block for 10 minutes at 95°C. The samples were centrifuged for 3 minutes at 15,000 x g and the supernatant (containing the DNA) was collected and used for the MG-LAMP assay. Five µL of the supernatant was used in the MG-LAMP assay and the rest stored in the -80ºC freezer. The assay was performed in a 20µL total reaction volume, which contained 2X in-house buffer (40mM Tris-HCL pH 8.8, 20mM KCl, 16mM $MgSO_4$, 20mM $(NH_4)SO_4$, 0.2% Tween-20, 0.8M Betaine, 2,8mM of dNTPs each), 0.004% MG, 8 units of *Bst* polymerase (New England Biolabs, Ipswich, MA) and 5µL of template DNA (Bell *et al*., 2016). Mitochondria *Plasmodium* genus-specific primers were used to amplify the DNA at 63^{o}C for 60 minutes using a simple heat block (Figure 3.2). The samples were then allowed to cool for 15 minutes before the results were scored by three independent readers by visual inspection of color change.

REGION	PRIMER SEQUENCE (5' to 3')
F1P (F1C+F2)	AGCTGGAATTACCGCGGCTGGGTTCCTAGAGAAACAATTGG
B1P (B1+B2C)	TGTTGCAGTTAAAACGTTCGTAGCCCAAACCAGTTTAAATGAAAC
F3	TGTAATTGGAATGATAGGAATTTA
B3C	GAAAACCTTATTTTGAACAAAGC
LPF	GCACCAGACTTGCCCT
LPB	TTGAATATTAAAGAA

Figure 3.2: Primer sequences used in the LAMP assay.

Positive (known *P. falciparum)* and negative (no template/DNA) control samples were included in each run. Positive samples were those that retained a light green/blue malachite green color while negative samples remained colorless. For quality control purposes, 20% of the samples were randomly selected and retested at KEMRI/CGHR Malaria Laboratories as recommended by the WHO.

3.6.4 Quantitative Real-Time PCR

The QIAamp DNA Mini Blood Kit (Quigen, Valencia, CA) was used to extract DNA from DBS prepared from 50µL of blood. Commercially available TaqMan Universal Master Mix (Applied Biosystems) was used. Species-specific probes corresponding to *P. falciparum* were used to detect the presence of *P. falciparum.* The Rougemont real-time PCR was performed using standard equipment and methods as previously described (Lucchi *et al*., 2013). Positive (known *P. falciparum* positive sample) and negative (no template/DNA) control sample were included in each run. All samples were run in duplicates. A threshold cycle number (Ct) of 40 was used as the cut-off in order to consider a sample positive or negative: all samples which did not amplify and those that amplified after a Ct value of 40 were considered negative and all samples that amplified before Ct value of 40 were considered positive.

3.7 Feasibility of using MG-LAMP in a local health facility

The feasibility of performing MG-LAMP assay as a diagnostic test in the local health facility was determined by the perceptions of the study participants and health workers, the turn-around time for obtaining patients' results, the cost of running the assay and the ease of training a local technician to perform the assay proficiently.

3.7.1 Turn-Around Time

The turn-around time of MG-LAMP, microscopy, RDT and qPCR was determined using calculating the time taken by the diagnostic tools from sample preparation to reporting of the results. The time taken was compared amongst the tests to determine which had the shortest turn-around time and which had the longest.

3.7.2 Health Workers' Perception Using MG-LAMP

Seven randomly selected health care workers, 5 based at Siaya Hospital (2 technologists, 1 nurse, 1 medical doctor and the laboratory manager) and 2 (1 technologist and 1 clinical officer) based at Rota Dispensary were selected. They were

interviewed using a structured questionnaire to know their opinion on the applicability of MG-LAMP at the two health facilities (Appendix V).

3.7.3 Study Participants' Perception of Using MG-LAMP

The selection of the study participants was done using systematic random sampling, whereby, the 12th participant was selected to participate in the interview. Therefore, 22 study participants (8 at Siaya Hospital and 14 at Rota Dispensary) were interviewed using a structured questionnaire to evaluate their perceptions about the MG-LAMP assay as a malaria diagnostic test (Appendix VI).

3.8 Data Management and Analysis

All data was collected using standardized forms and questionnaires (Appendix V and VI) and entered into Microsoft Excel (Microsoft Corp, Redmond, Washington, USA). Data analysis was carried out using Stata version 14.2 (StataCorp, College Station, TX, USA). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were determined for rapid diagnostic test (RDT), routine microscopy, expert microscopy and MG-LAMP, using qPCR as the reference standard as previously described by Lucchi *et al*. (2016).

$$\text{Sensitivity} = \frac{\text{number of true postives}}{\text{number of true positives} + \text{number of false negatives}} \times 100$$

$$\text{Specificity} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false positives}} \times 100$$

$$\text{PPV} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false positives}} \times 100$$

$$\text{NPV} = \frac{\text{number of true negatives}}{\text{number of true negative} + \text{number of false negative}} \times 100$$

Kappa coefficient was also calculated to assess the agreement among the different diagnostic methods. P value below 0.05 was considered statistically significant.

3.9 Ethical approval

Ethical approval was obtained from the Kenyatta University Ethics Review Committee. Ethical approval was obtained before the commencement of the study. This study involved indirect contact with the patients, therefore, no harm or pain was

anticipated for the patients within the auspices of this study. Samples were received in special codes and would then be assigned new codes for the purposes of the study. Data was also protected with a password, which was only available to the investigator and the supervisors.

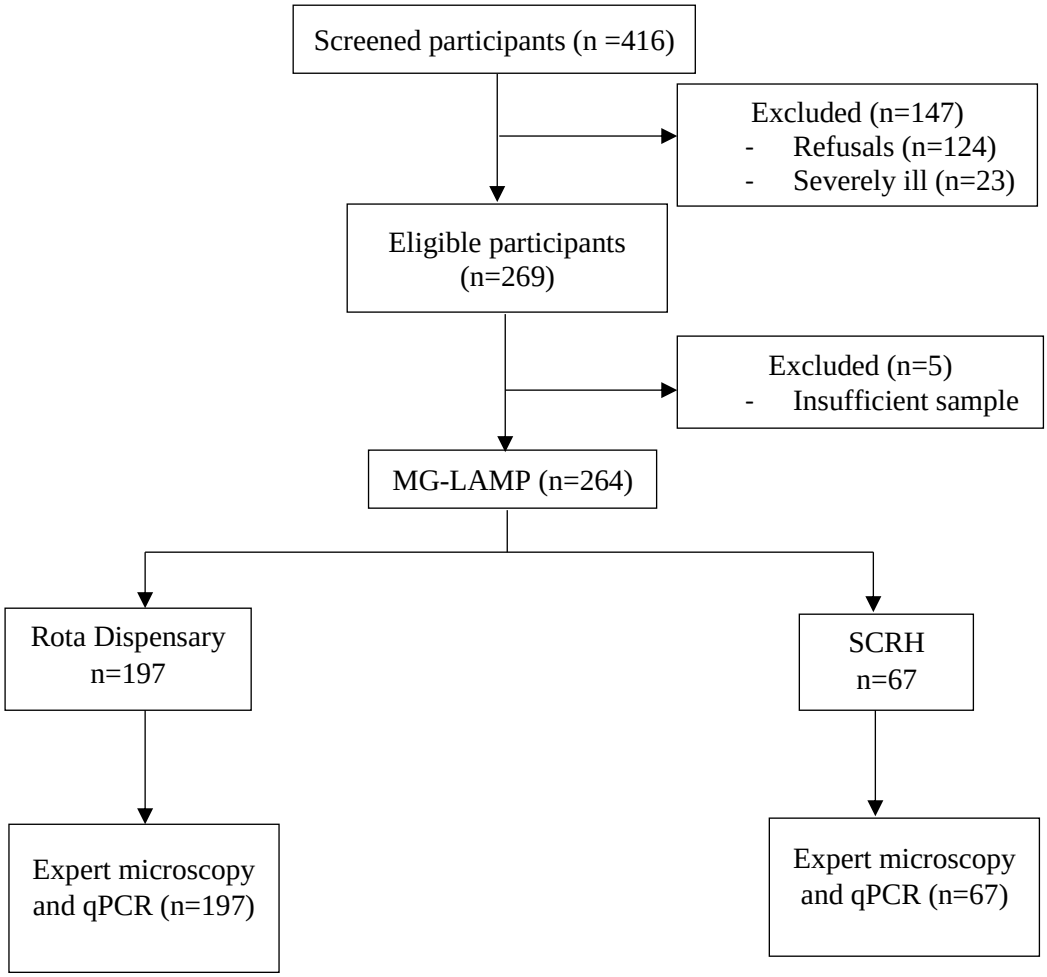

Figure 3.3: Malaria diagnosis at the two health facilities.

Note; SCRH: Siaya County Referral Hospital.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics of Study Participants

A total of 416 patients presenting to the two health facilities with suspected malaria were screened for enrollment into the study. Two hundred and sixty-four were enrolled, 197 at Rota Dispensary and 67 at Siaya Hospital and one hundred and fifty-two participants were excluded for various reasons.

The characteristics of the study participants are shown on Table 4.1. There was no significant difference in gender and mean parasite densities by expert microscopy for participants enrolled at the two health facilities. However, participants enrolled at Rota Dispensary were older, 16.8 years (range 6 months-62 years) compared to those who were enrolled at Siaya Hospital, 7.2 years (range 8 months-51 years).

Table 4.1: Demographic Characteristics of Study Participants

Characteristic	Rota Dispensary N=197	Siaya Hospital N=67	P-Value
Gender, n (%)			
Male	71 (36)	28 (42)	0.3809
Female	126 (64)	39 (58)	0.3809
Age, years (R)	16.8 (6months-62 years)	7.2 (8months-51 years)	0.0000
Mean parasite density, P/µL (R)	84638 (0-1005163)	69250 (0-473175)	0.6422

4.2 Malaria Positivity by RDT, Routine Microscopy, Expert Microscopy, MG-LAMP and qPCR

At Rota Dispensary, where RDTs are used for malaria diagnosis, the malaria positivity by RDT, MG-LAMP, qPCR and expert microscopy were comparable, 37%, 44%, 42%

and 30% respectively. Similarly, at Siaya Hospital where microscopy is used for routine diagnosis, there was no significant difference in positivity rate by routine microscopy, MG-LAMP, qPCR and expert microscopy (Figure 4.1).

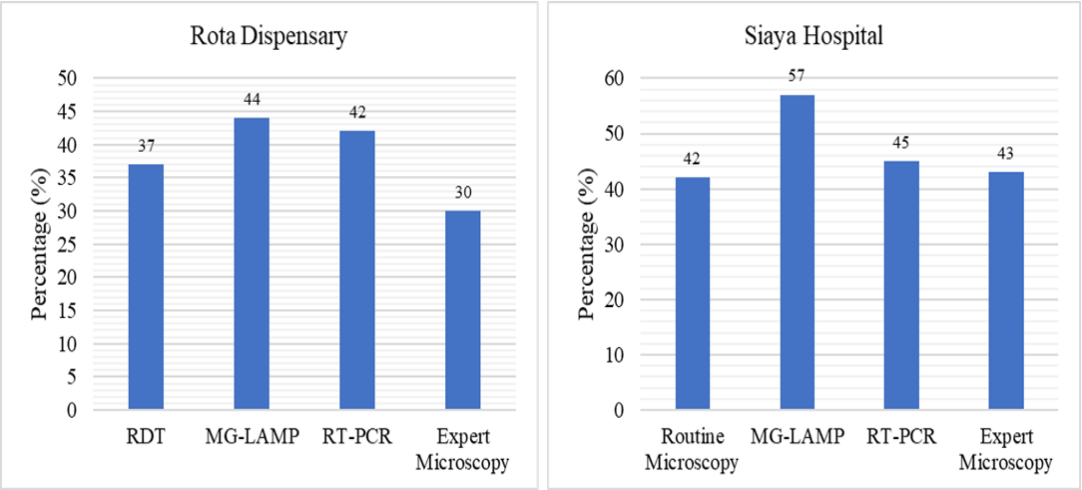

Figure 4.1: Malaria positivity by RDT, routine microscopy, MG-LAMP, qPCR and expert microscopy at Rota Dispensary and Siaya Hospital.

4.3 Discordant Results Between RDT, Routine Microscopy, Expert Microscopy, MG-LAMP and qPCR

The study determined the number of patients identified as positive or negative by RDT, routine microscopy, expert microscopy, MG-LAMP and qPCR at the two health facilities (Table 4.2). At Rota dispensary, there were a total of 15 participants who were positive by RDT but negative by MG-LAMP (14), qPCR (8) and expert microscopy (15). At the same health facility, a total of 29 participants were positive by MG-LAMP (29), qPCR (18) and expert microscopy (2) but negative by RDT. At Siaya Hospital, there was agreement between routine and expert microscopy on the number of positive participants but the results of 3 were discordant and scored as positive by routine microscopy but negative by MG-LAMP (3) and qPCR (3). At the same health facility, a total of 13 participants were positive by MG-LAMP (13), qPCR (5) and expert microscopy (1) but negative by routine microscopy.

Table 4.2: Summary of Discordant Results at Rota Dispensary and Siaya County Referral Hospital

Rota Dispensary				
Method	**RDT Positive, n=72**		**RDT Negative, n=125**	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
MG-LAMP	58 (80.6)	14 (19.4)	29 (23.2)	96 (76.8)
qPCR	64 (88.9)	8 (11.1)	18 (14.4)	107 (85.6)
Expert Microscopy	57 (79.2)	15 (20.8)	2 (1.6)	123 (98.4)
Siaya Hospital				
	[a]R-Microscopy Positive, n=28		**R-Microscopy Negative, n=39**	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
MG-LAMP	25 (89.3)	3 (10.7)	13 (33.3)	26 (66.7)
qPCR	25 (89.3)	3 (10.7)	5 (12.8)	34 (87.2)
Expert Microscopy	28 (100)	0 (0)	1 (2.6)	38 (97.4)

Note: [a] = Routine Microscopy

4.4 Performance Characteristics of RDT, Routine Microscopy, Expert Microscopy and MG-LAMP using qPCR as a Reference Standard

The sensitivity, specificity, positive and negative predictive values of the different diagnostic tests are shown in Table 4.3. At Rota dispensary, the sensitivity of RDT, MG-LAMP and expert microscopy was 78.1% (CI 67.5-86.4), 82.9% (CI 73.0-90.3) and 72.0% (CI 61-81.3) respectively. At the same facility, the specificity for the three diagnostic tests was 93.0% (CI 86.8-97), 83.5% (CI 75.4-89.8) and 100% (CI 96.8-100) respectively. At Siaya County Referral Hospital, the sensitivity of routine microscopy, MG-LAMP and expert microscopy was 83.3% (CI 65.3- 94.4), 93.3% (CI 77.9-99.2) and 86.7% (CI 69.3-96.2) respectively. At this facility, the specificity for

the three diagnostic tests was 91.9% (CI 78.1-98.3), 73.0% (CI 55.9-86.2) and 91.9% (CI 78.1-98.3) respectively.

Table 4.3: Sensitivity, Specificity, Positive and Negative Predictive Values of RDT, Routine Microscopy, MG-LAMP and Expert Microscopy Using qPCR as Reference Standard.

Rota Dispensary					
Method	**Sensitivity % (CI)**	**Specificity % (CI)**	**PPV % (CI)**	**NPV % (CI)**	**K-Value % (CI)**
RDT	78.1 (67.5-86.4)	93.0 (86.8-97)	88.9 (79.3-95.1)	85.6 (78.2-91.2)	0.72 (0.59-0.86)
MG-LAMP	82.9 (73.0-90.3)	83.5 (75.4-89.8)	78.1 (68.0-86.3)	87.3 (79.6-92.9)	0.66 (0.52-0.80)
E-Microscopy	72.0 (61-81.3)	100 (96.8-100)	100 (93.9-100)	83.3 (78-87.6)	0.75 (0.61-0.88)
Siaya Hospital					
R-Microscopy	83.3 (65.3-94.4)	91.9 (78.1-98.3)	89.3 (71.8-97.7)	87.2 (72.6-95.7)	0.76 (0.52-1.00)
MG-LAMP	93.3 (77.9-99.2)	73.0 (55.9-86.2)	73.7 (56.9-86.6)	93.1 (77.2-99.2)	0.65 (0.41-0.88)
E-Microscopy	86.7 (69.3-96.2)	91.9 (78.1-98.3)	89.7 (74.4-96.3)	89.5 (77.3-95.5)	0.79 (0.55-1.03)

Note: R-Microscopy = Routine microscopy, E-Microscopy=expert microscopy, PPV=positive predictive value, NPV=negative predictive value, CI=Confidence Interval

4.5 The Performance Characteristic of MG-LAMP at Different Parasite Densities

The parasite densities were grouped into five groups according to qPCR positive results (n=112). The five groups included <50 P/µl (n=23), 50-200 P/µl (n=8), 200-500 P/µl (n=5), 500-2000 P/µl (n=8) and >2000 P/µl (n=68). The sensitivity of MG-LAMP was 47.8%, 87.5%, 100%, 100% and 95.6% respectively, (Figure 4.3). In all the parasite densities, MG-LAMP had a positive predictive value of 100%.

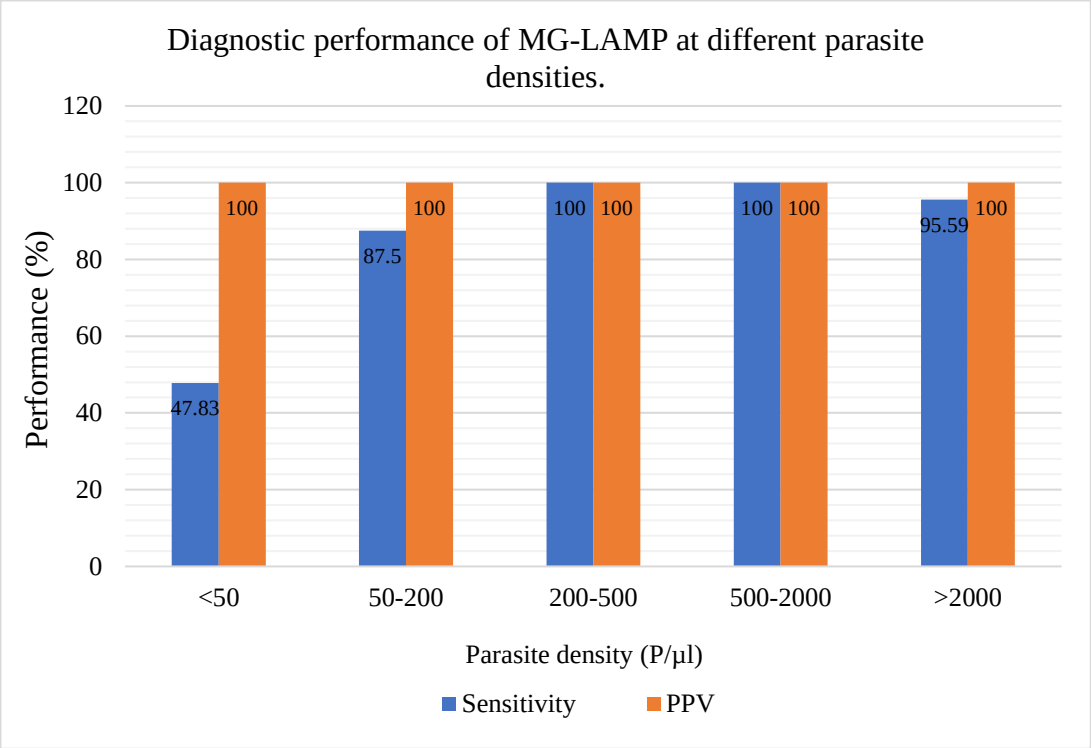

Figure 4.2: Sensitivity of MG-LAMP at different parasite densities using qPCR as the reference standard.

4.6 Feasibility of Using MG-LAMP in Health Facilities

4.6.1 Turn-around Time of MG-LAMP Assay

The turn-around-time of different malaria diagnostic tools (microscopy, RDT, qPCR and MG-LAMP) was compared from the time of samples preparation to the time of testing and reporting. Malaria RDT had the shortest turn-around-time of 20 minutes while microscopy had a time of 60 minutes and MG-LAMP had a turn-around-time of 105 minutes. Quantitative PCR had the longest turn-around time of 270 minutes, (Table 4.4).

Table 4.4: Turn-around Time of Microscopy, RDT, qPCR and MG-LAMP Assay

	Time in minutes		
Diagnostic tests	**Sample preparation**	**Testing and reporting**	**Total time**
Microscopy	50	10	60
RDT	5	15	20
qPCR	180	90	270
MG-LAMP	30	75	105

4.6.2 Health Workers and Study Participants Opinion on the Use of MG-LAMP in Health Facilities

When interviewed using a structured questionnaire, 6 out 7 health workers (2 at Rota Dispensary and 4 at Siaya Hospital) and 8 out 22 of study participants (5 at Rota Dispensary and 3 at Siaya Hospital) agreed that MG-LAMP can be used in health facilities for malaria diagnosis. One health worker and 14 study participants (9 at Rota Dispensary and 5 at Siaya Hospital) disagreed that MG-LAMP can be used as a routine diagnostic test for patient management in a health facility.

CHAPTER FIVE

DISCUSSION

5.1 Discussion

The WHO recommends the universal diagnosis of all suspected malaria cases using quality-assured microscopy and RDTs (WHO-FIND-CDC, 2018). Due to weak or non-existence of QA/QC systems in endemic areas to support this recommendation, it is important to periodically check the performance of routine diagnostic methods and whether the results compare with those of more sensitive methods such as expert microscopy and molecular methods. In this study, diagnostic results obtained by routine RDTs and smear microscopy at two different levels of health care facilities in an area of high and perennial malaria transmission of western Kenya were compared with results obtained by expert microscopy and more sensitive molecular diagnostic methods; a simple calorimetric-based LAMP and qPCR.

There was no significant difference in malaria positivity rate by routine RDT and microscopy at the two health facilities and the comparative diagnostic methods-expert microscopy, qPCR and MG-LAMP at the two health facilities. The malaria positivity rate by RDT at Rota Dispensary was slightly higher than the other diagnostics tests used in this study. This is similar to what has been reported in previous studies. In an analysis of 85,000 children enrolled in Demographic and Health Surveys and Malaria Indicator surveys across 15 countries in sub-Saharan Africa, the mean malaria prevalence was 24.5% by microscopy and 30.3% by RDTs (Watson *et al*., 2019). Similar positivity between RDTs have been shown in São Tomé, 37% by microscopy and 53% by RDT (Shaio *et al*., 2012). In Tanzania, similar results have been reported whereby malaria positivity was 57.9% by RDT and 52% by microscopy (Bwire *et al*., 2019), and in Cameroon, 31% by microscopy and 45% by RDT (Mfuh *et al*., 2019). The higher positivity by RDTs compared to microscopy can be attributed to the persistence of antigens detected by HRP-2 in the blood for longer periods even after treatment or past infection (Waitumbi *et al*., 2011). This is a challenge for health managers who use results based on RDTs only to estimate malaria case burdens and thus the need for robust secondary diagnostic methods or QA/QC systems in settings where RDTs are the main diagnostic methods. Similar to what has been reported in

previous studies that have compared the diagnostic performance of RDTs, routine microscopy and molecular tests, the positivity rates by molecular tests were higher (Masanja *et al*., 2015; Mfuh *et al*., 2019; Waitumbi *et al*., 2011; Wanja *et al*., 2016). This is not surprising since the threshold of parasite detection for the molecular tests is significantly lower, 1-2 parasites/µL for PCR assays compared to RDTs (100-200 parasites/µL) and microscopy (Tambo *et al*., 2018).

In this study, 29 and 13 patients at Rota Dispensary and Siaya Hospital respectively, were negative by the routine diagnostic tests used at these health facilities but they were positive by MG-LAMP, qPCR and expert microscopy. This implies that these patients had malaria but were not treated. In the absence of differential diagnosis such as access to blood cultures or PCR to rule out causes of clinical symptoms at many public health facilities in endemic areas, especially in young children, untreated *P. falciparum* malaria can progress rapidly to severe and life-threatening forms of the disease (ACTwatch Group *et al*., 2017). This can lead to deaths and undermine both the clinical confidence and credibility of health services and health facility data if patients who might have malaria are not treated and the cause of symptoms for the hospital visit is not identified (Hailu *et al*., 2017). Additionally, untreated cases can contribute to the transmission of malaria in an area (Beshir *et al*., 2013). The study also found 15 positive cases by RDT but negative by MG-LAMP, qPCR and expert microscopy at the dispensary and 3 cases which were positive by routine microscopy but negative by MG-LAMP and qPCR at Siaya Hospital. According to WHO, both microscopy and RDTs must be supported by a quality assurance programme (WHO, 2015). This reduces the chance of misdiagnosis and improves patient management. Previous studies have also reported discrepancy between different diagnostic methods (Kudyba *et al*., 2019; Morris *et al*., 2017; Oriero *et al*., 2015). Since NAATs diagnostic methods are more sensitive and have a lower limit of parasite detection than RDTs and microscopy, discrepancies are expected in the results obtained by NAATs and other diagnostic methods. Previous studies have reported a limit of detection of <6 parasites/µL for NAATs compared to 100-200 parasites/µL for RDTs and 50 parasites/µL for microscopy (Cheaveau *et al*., 2018; WHO, 2014). The sensitivity of MG-LAMP was higher than RDT and microscopy, but a lower specificity at the two

health facilities. These results are consistent with previous studies showing the higher sensitivity but lower specificity of MG-LAMP compared to microscopy and RDT (Björkman *et al*., 2015; Kudyba *et al*., 2019; Vincent *et al*., 2018). There are many factors which could affect the sensitivity of different diagnostic methods including sample collection method and preparation, efficiency of nucleic acid extraction procedure, amount of blood, amount of template used in the reaction, copy number of target sequence and the buffers, enzymes and other materials used (WHO, 2014). However, these limitations can be overcome by standardization of the methods and use of quality-assured reagents.

The sensitivity of MG-LAMP in relation to parasite density was shown to increase as the parasite density increases, with the PPV at 100%. The MG-LAMP assay was able to detect malaria parasites at parasite density of below 50 parasites/µl, indicating that it is capable of detecting low-malaria density infections, below the threshold of microscopy and RDTs. At above 2000 parasites/µl, the sensitivity of MG-LAMP decreased to 95.6%, indicating that the assay is affected by high parasite densities. Hopkins *et al*. (2013) demonstrated that LAMP had sensitivity of 66% at parasite density <1 P/µL, 97.8% at ≥2 P/µl and 97.5% at ≥5 P/µL stratified by quantitative PCR determined parasite density. Although the sensitivity of MG-LAMP decreased (95.6%), it was within the WHO recommended sensitivity of above 95%.

The MG-LAMP evaluated in this study was simple and can be used by health care providers without previous training in molecular methods. Compared to the health workers, more than half of the study participants interviewed disagreed that MG-LAMP can be used in a health facility setting. This was mainly because of the turn-around-time that was needed to obtain results. This shows a disconnect between health workers and community members when it comes to diagnostic tests. Patients prefer tests that have a short turn-around time, implying short stay at the health facility, while health workers might prefer tests that are more sensitive (Altaras *et al*., 2016; Mokuolu *et al*., 2018). Another drawback for the MG-LAMP assay is that the equipment used for DNA isolation process (water bath and centrifuge) require electricity which might be a challenge in resource-limited settings and thus limiting its use at the community level and at lower levels of the healthcare system.

There are several reports highlighting the challenges of malaria diagnostic tests in endemic areas (Diallo *et al*., 2018; Jemere *et al*., 2018; Kabaghe *et al*., 2017; Odhiambo *et al*., 2017; Wanja *et al*., 2017; Zimmerman & Howes, 2015). Despite these shortcomings, RDTs and microscopy procedures that are not quality assured continue to be used in many malaria endemic areas. This could result in misdiagnosis leading to inappropriate patient management, irrational use of antimalarial drugs and generation of inaccurate data on the malaria burden (UNITAID, 2015). Therefore, there is a need to strengthen the quality assurance processes for malaria diagnostics using inexpensive and novel strategies such as placing simple, inexpensive and more sensitive molecular diagnostic assays at regional centres to strengthen the national QA/QC programmes.

This study has several limitations. There was a selection bias since only patients who presented to the health facilities with symptoms suggestive of malaria were enrolled. However, since the amin objective of the study was to evaluate the performance of routine diagnostic methods at two different levels of heath care system, these results could reflect the performance of the diagnostics tests evaluated at health facilities in malaria endemic areas. Another limitation is comparison of results using diagnostic methods that use different input samples that is extraction of DNA from DBS versus whole blood. However, these methods are well standardized and are used widely for malaria diagnosis for different objectives such as clinical management-RDTs and microscopy-research and in elimination settings-NAATs. Another limitation is the small sample size at the Siaya hospital where only 67 participants were enrolled. Patients attending a referral hospital are typically sicker and likely referred from either a dispensary or health center. Therefore, the results from the dispensary where a larger number of participants were enrolled compensates for the low numbers enrolled at the referral hospital. A major limitation of the LAMP assay is the inability to quantify parasite density. Since the objective of this study was to evaluate whether LAMP can be used as a reference method for RDTs and microscopy in a QA/QC program, this might not be a major drawback since it is more sensitive than RDTs and microscopy.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The MG-LAMP evaluated in this study is a simple and sensitive assay in the detection of malaria parasites compared to RDTs and microscopy which are used for routine malaria diagnosis at health facilities. This makes it an inexpensive reference test in a quality control tool to monitor the performance of RDTs and smear microscopy in resource-limited settings. This will improve both patient management of suspected malaria cases and the quality of health facility surveillance data.

The MG-LAMP assay could be used at resource-limited health facilities as it is simple and easy to perform and ease of training of the healthcare workers on how to use it. Although the assay can be applied at resource-limited health facilities, it still requires electricity for it to run. This makes it difficult to use the MG-LAMP in health facilities without electricity.

6.2 Recommendations

Compared to microscopy, the LAMP assay is still expensive ($16). Therefore, further refinement of the assay is required so as to reduce the cost of running the assay close to the WHO recommendation of $5, which is the cost running malaria rapid diagnostic tests. This refinement of the assay will also help shorten the turn-around time of the assay as the turn-around time is still long compared to that of microscopy. Additionally, the DNA isolation step of the assay needs to be improved, to reduce the chance of contamination and reduce the use of equipment that require electricity, making more applicable in remote malaria endemic areas.

Further research on the perceptions of using the MG-LAMP assay at local health facilities should be done at the national level so as to know and understand the consumer needs in relation to the diagnostic assay.

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APPENDICES

Appendix I: Informed Consent Form (Flesh-Kincaid 7.2) for patients ≥ 18-years-old

Project: Evaluation of the colorimetric malachite green loop-mediated isothermal amplification (MG-LAMP) assay for the detection of malaria species at two different health facilities in a malaria endemic area of western Kenya.

Purpose

The Centers for Disease and Control and Prevention (CDC) of the United States and the Kenya Medical Research Institute (KEMRI) want to find better ways to test for malaria. We would like to invite you to take part in this evaluation.

Evaluation methods

We want to know how well new tests for malaria will work. To do this, we need to do some tests to see if you have malaria.

To use the malaria test, we will take a few drops of blood from your finger. If you agree to take part in this evaluation, we will take about 300uL of blood from a finger stick. This will be used to check for malaria using RDT, microscope and a new test that we are evaluating. We will not use the blood for testing any other illness. The test results from the test that we are doing will not be used to make decision about your treatment.

We will also ask some questions about you, like your age, how long you have been sick, what you think about a new test for malaria, how long you would be willing to wait for your results from this test.

The tests will be free of charge. The interview and blood collection will take about 15 minutes.

Benefits and Risks

You will not have any direct benefit from the testing that we will do for a new malaria test. However, your participation will help to evaluate a new test to detect malaria infection.

What we learn during this evaluation will help us to improve the diagnosis of malaria in the future.

You may feel a brief moment of pain or fear as your finger is pricked. Rarely some bruising or infection may happen at the site of prick.

You do not need to answer any question that you do not want to. Also, you can decide at any time you do not want to take part in this evaluation. You do not have to agree to us taking some blood from your finger.

Compensation

You will not receive any compensation for participating in this evaluation.

Privacy

We will keep your information confidential to the full extent permitted by the law. Other than the evaluation team, we will keep any facts you give us private to the extent the law allows. All records from this evaluation will be locked away. Only the evaluation team will have access to them. We will not use your name in any report that comes from this evaluation.

Contact information

If you have any question or if any problems remain unsolved, contact:

- Dr. Simon Kariuki at KEMRI/CDC, Box 1578, Kisumu, or on Telephone 057 20 22902. You can also contact any of our Clinical Officers at a nearby health centre. If you have any questions about your rights as a study participant, or if you want to talk about the study with someone who is not directly involved with this study, please contact The Secretary, KEMRI Ethics Review Committee, P.O. Box 54840-00200, Nairobi; Telephone numbers: 020 2722541, 0722205901 or 0733400003. Email address: erc@kemri.org.

Voluntary participation, refusal and withdrawal

Several aspects of this evaluation are very important. Remember that:

- You are free to decide whether or not to take part in this evaluation. You will not incur any penalty or loss for not taking part.

- You are free to withdraw from this evaluation at any time. Your decision will not affect your treatment or the care received.
- There is no cost to you for taking part in this evaluation.
- You may let me know now if you have any questions with respect to the evaluation. You can also ask questions about your participation.

Declaration of consent

Upon signing the appropriate declaration, I agree that:

- I have read this form or the form was read to me by someone else
- I was able to ask questions about the evaluation. My questions have been answered
- I agree in a voluntary manner to participate in this evaluation
- I was informed that I have the right to withdraw from the evaluation at any time. That will not affect the care given to me or my child

Name of the responsible party: ……... Evaluation ID Number: ……

Signature of the responsible party……... Date…….

Fingerprint ……

I bear witness to the informed consent and can verify that the participant or responsible party was informed of the details, risks and benefits of the assessment, and had the opportunity for their questions to be answered.

Name of witness: …….

Signature of Witness …… Date……...

Long-term storage of samples for future tests

Some of the collected blood may be left over after this evaluation is completed. We would like to store any leftover blood. These will be used for future studies relating only to malaria.

You may still take part in this evaluation even if you do not wish for the long-term storage of your blood for future use. In that case, your blood will be destroyed at the end of this evaluation. Even if you do not allow storage of the sample for future use, you will receive the usual medical care.

I give my permission for the storage of my blood for future use. ( )

I do not give my permission to store my blood for future use. ( )

Name of the responsible party: ……... Evaluation ID Number: ……

Signature of the responsible party…… Date……...

Fingerprint……

Declaration by investigator

I have adequately read, or born witness to the exact reading of the consent form, to the participant and the individual had the opportunity to ask questions. I confirm that the individual freely gave their consent.

Printed name of the member of the assessment team …….

Signature of the member of the assessment team……

Note: Three copies will be made of this consent. One will remain with the patient or individual responsible for him or her, another will remain on file at the institution where the research takes place, and the third will remain with the coordinator of this assessment.

Appendix II: Assent Form (for participants aged 13-17 years) (Flesch-Kincaid Readability 4.5)

Project: Evaluation of the colorimetric malachite green loop-mediated isothermal amplification (MG-LAMP) assay for the detection of malaria species at two different health facilities in a malaria endemic area of western Kenya.

Purpose

The Centers for Disease and Control and Prevention (CDC) of the United States and the Kenya Medical Research Institute (KEMRI) want to find better ways to test for malaria. Your parents/ guardian agreed that you can take part. It is your choice to decide to take part in this evaluation. You do not have to be part of the evaluation if you do not want to.

We would like to ask you to participate in this evaluation. We want to find better ways to verify if you have malaria.

If you agree, we will take about 300uL of blood from your finger. We will use this blood to test if you have malaria. We will ask you some questions about your health and what you think about a new test for malaria. This will take about 15 minutes.

It may hurt when we prick your finger. Some bleeding or an infection may occur. The persons doing the collection of blood are very well trained. They will use a safe method for taking blood. This reduces risk of bleeding or local infection.

You can choose if you want to join or not. It is okay if you do not want to join. In that case, the attendant at the diagnostic post will still see you. You can also change your mind at any time.

You can ask your mother or father if you have any questions. You can also ask questions to the person at the diagnostic post, or any of the researchers.

Do you have any questions?

Your mom, dad or guardian said that it is all right to be in the evaluation. Please let me know if you would like to take part.

Name of child: ……. Evaluation identification Number: ……

Signature of the child……... Date…….

Appendix III: Consent Form for the children < 18 years old, care-giver consent

Informed Consent Form (Flesh-Kincaid 7.2)

Project: Evaluation of the colorimetric malachite green loop-mediated isothermal amplification (MG-LAMP) assay for the detection of malaria species at two different health facilities in a malaria endemic area of western Kenya.

Purpose

The Centers for Disease and Control and Prevention (CDC) of the United States and the Kenya Medical Research Institute (KEMRI) want to find better ways to test for malaria. We would like to invite your child to take part in this evaluation.

Evaluation methods

We want to know how well new tests for malaria will work. To do this, we will use tests to see if your child has malaria.

To use the malaria test, we will take a few drops of blood from your child's finger. If your child agrees to take part in this evaluation we will take about 300uL of blood from his/her finger. This will be used to check for malaria using, RDT, microscope and a new test. We will not use the blood for testing any other illness.

We will also ask some questions about your child for example, your child's age, how long your child has been sick, what your child thinks about a new test for malaria, how long she/he would be willing to wait for results from this test.

The tests will be free of charge. The interview and blood collection will take about 15 minutes.

Benefits and Risks

Your child will not receive any benefit from this evaluation to make a new malaria test. However, your child's participation will help to evaluate a new test to detect malaria infection.

What we learn during this evaluation will help us to improve the diagnosis of malaria in the future.

Your child may feel a brief moment of pain or fear as his/her finger is pricked. Rarely some bruising or infection may happen at the site of prick.

You do not need to answer any question that you do not want to. Also, you can decide at any time if he/she will take part in this evaluation. You do not have to agree to us taking some blood from your child's finger. Your child health care will not change from normal if you choose not to answer or to have blood taken.

Compensation

You will not receive any compensation for participating in this evaluation.

Privacy

We will keep your or your child's information confidential to the full extent permitted by the law. Your name, or that of your child, will be used only during the time you come for your appointment. Other than the evaluation team, we will keep any facts you give us private to the extent the law allows. All records from this evaluation will be locked away. Only the evaluation team will have access to them. We will not use your child's name in any report that comes from this evaluation.

Contact information

If you have any question or if any problems remain unsolved, contact:

- Dr. Simon Kariuki at KEMRI/CDC, Box 1578, Kisumu, or on Telephone 057 20 22902. You can also contact any of our Clinical Officers at a nearby health centre. If you have any questions about your rights as a study participant, or if you want to talk about the study with someone who is not directly involved with this study, please contact The Secretary, KEMRI Ethics Review Committee, P.O. Box 54840-00200, Nairobi; Telephone numbers: 020 2722541, 0722205901 or 0733400003. Email address: erc@kemri.org.

Voluntary participation, refusal and withdrawal

Several aspects of this evaluation are very important. Remember that:

- You are free to decide whether or not your child will take part in this evaluation. If she/he decide not to take part, or your child will receive the

necessary free routine treatment, for malaria. She/he will not incur any penalty or loss for not taking part.

- You are free to withdraw him/her from this evaluation at any time. That fact will not affect your child's treatment or the care received.
- There is no cost to your child for taking part in this evaluation.
- You may let me know now if you have any questions with respect to the evaluation. You can also ask questions about your or your child's, participation.

Declaration of consent

Upon signing the appropriate declaration, I agree that:

- I have read this form or the form was read to me by someone else
- I was able to ask questions about the evaluation. My questions have been answered
- I agree in a voluntary manner to let my child participate in this evaluation
- I was informed that I have the right to withdraw my child from the evaluation at any time. That will not affect the care given to my child

Name of Participant: ……..…… Evaluation ID Number: ……

Signature of the responsible party……...…… Date……...

Fingerprint

I bore witness to the informed consent and can verify that the participant or responsible party was informed of the details, risks and benefits of the assessment, and had the opportunity for their questions to be answered.

Signature of Witness 1…….

Date…….

Long-term storage of samples for future tests

Some of your child's blood may be left over after this evaluation is completed. We would like to store any leftover blood. This blood will be used for future studies relating only to malaria.

Your child may still take part in this evaluation even if you do not wish for the blood to be stored for future use. In that case your child's blood will be destroyed at the end of this evaluation. Even if you do not allow storage of the blood for future use your child will receive the usual medical care.

I give my permission for the storage of my child's blood specimen, for future use. ( )

I do not give my permission to store my child's blood specimen, for future use. ( )

Name of Participant: ……..…… Evaluation ID Number: ……

Signature of the responsible party…….…

Date…….

Fingerprint……

Declaration by investigator

I have adequately read, or born witness to the exact reading of the consent form to the relative (mother or father) of the participant, or respective guardian, and the individual had the opportunity to ask questions. I confirm that the individual freely gave their consent.

Printed name of the member of the assessment team……

Signature of the member of the assessment team…….

Note: Three copies will be made of this consent. One will remain with the patient or individual responsible for him or her, another will remain on file at the institution where the research takes place, and the third will remain with the coordinator of this assessment.

Appendix IV: Case Record Form

Date: _____ Evaluation Number/Study (ID)_____

Village: _____ Participant's Age: _____ Yrs

Participant's DOB (DD-MMM-YYYY): _____

Participant's Sex: Male ( )

Female ( )

Temperature: __ __. __ ºC

Have you had fever in the last 24 hours?

( ) Yes ( ) No

Have you taken any anti-malarial medication for the last one month?

( ) Yes ( ) No

If yes, what type of anti-malarial medication did you take? _____

Blood obtained for MG-LAMP?

( ) Yes ( ) No

Lab Number _____ Time of sample collection: __ __. __ __ hrs

What amount of blood collected? _____

If less sample is collected, prioritize as follows:

(1) MG-LAMP assay (2) PCR (3) BS (4) Other RDTs

Dry Blood Spots on Filter paper obtained?

( ) Yes ( ) No

If yes, how many blood spots taken? _____

Malaria Care Start Pf HRP2/pLDH RDT performed?

( ) Yes ( ) No

Malaria First Response Ag Pf HRP2/pLDH Combo performed?

( ) Yes ( ) No

Blood smear prepared?

( ) Yes ( ) No

Result of the RDT done at the health facility for malaria diagnosis:

( ) Positive ( ) Negative ( ) Indeterminate ( ) Not applicable

Which RDT kit is the health facility using?

( ) Carestart Pf HRP2

( ) Carestart Pf HRP2/pLDH

( ) First response Ag Pf HRP2/pLDH Combo

( ) SD Bioline Malaria Ag P.f/Pan

Result of microscopy test done at the health facility for malaria diagnosis:

( ) Positive ( ) Negative ( ) Not applicable

Parasite density: _____/uL or other reporting system _____

Reviewed By: _____ Signature: _____ Date: _____

Appendix V: Healthcare Providers Perception of Malachite Green Loop-Mediated Isothermal Amplification (MG-LAMP) Assay

INSTRUCTIONS: ***Please, Tick Where Applicable***

Name of the Health Facility_____

Name/code or ID of the healthcare provider_____

Position in the Health Facility_____

Gender: Male [ ] Age: _____

Female [ ]

1a) What is your level of education?

[ ] Some primary

[ ] Complete primary

[ ] Some secondary

[ ] Complete secondary

[ ] Post-secondary education (certificate, diploma, graduate)

1b) Years of Experience?

[ ] Less than 1year

[ ] 1-2years

[ ] 3-4years

[ ] 4-5 years

[ ] More than 5years

1c) What methods are used for parasitological diagnosis in the health facility?

[ ] Microscopy

[ ] Rapid Diagnostic Tests (RDTs)

[ ] None

[ ] Other (Specify)_____

1d) Who performs malaria diagnosis in the health facility?

[ ] Clinician

[ ] Nurse

[ ] Laboratory personnel

[ ] Other (Specify) _____

1e) What other methods of malaria diagnosis are you aware of?

[ ] Quantitative Buffy Coat (QBC)

[ ] Serological tests

[ ] Molecular tests

[ ] None

[ ] Other (Specify) _____

2a) Which malaria molecular tests do you know?

[ ] Polymerase chain reaction (PCR)

[ ] Loop mediated isothermal amplification (LAMP)

[ ] Other (Specify) _____

2b) What are the advantages of molecular methods for malaria diagnosis?

[ ] High sensitivity and specificity

[ ] Short turnaround time

[ ] Does not require electricity

[ ] Can be performed by any health care worker including Community Health Volunteers (CHVs)

[ ] Does not require sophisticated equipment

[ ] None

[ ] Other (Specify)

_____

2c) What are the disadvantages of molecular methods for malaria diagnosis?

[ ] Requires trained personnel

[ ] Not useful in patients who have already taken antimalarial

[ ] Instructions on the insert are not clear

[ ] False negative and false positive results

[ ] None

[ ] Other (Specify)

_____

NOTE: Briefly describe the LAMP method to the healthcare provider.

Malachite-Green Loop Mediated Isothermal Amplification Assay

Introduction

Loop-mediated isothermal amplification (LAMP) is an isothermal DNA amplification method which was developed in 1998 by Eiken Chemical Company, Japan. LAMP requires four to six different primers (F1P, B1P, F3, B3, LPF, and LPB), that are designed specifically to recognize six to eight specific gene sequences. DNA amplification is accomplished with the use of DNA polymerase with strand-displacing activity. This strand-displacing activity allows amplification under isothermal

conditions in contrast to polymerase chain reaction (PCR) where a thermal denaturation step is essential.

Procedure

Fifty microliters of the collected blood will be used to extract DNA for the RealAmp assay. DNA will be released from the whole blood by boiling the blood in a heat-block at 95°C for 10 minutes. The tubes will be spun down using a centrifuge for 5 minutes and the supernatant (containing the DNA) will be collected and used in the LAMP assay. Five microliters of this supernatant will be used in the LAMP assay. The Malachite Green (MG) malaria LAMP assay will be performed in a 20µL total reaction volume, 0.004% MG, 8 units of Bst polymerase and 5µL of template DNA. Mitochondria Plasmodium genus-specific primers will be used to amplify the DNA. The amplification will be carried out at 63°C for 60 minutes using a simple heat- block. Samples will then be allowed to cool for 15 minutes before being scored by three independent human readers. Positive samples retain a light green/blue malachite green color while negative samples turn colorless.

(Ask the healthcare provider to carry out the LAMP assay)

3a) Is the loop-mediated isothermal amplification method easy to perform?

Yes [ ] No [ ]

3b) What is your opinion about the turnaround time for LAMP method for malaria diagnosis?

[ ] Long

[ ] Short

[ ] Satisfactory

[ ] Not applicable

3c) How much do you think the LAMP method would cost for malaria diagnosis?

[ ] Less than KSh100

[ ] KSh100 to KSh500

[ ] KSh500 to KSh1000

[ ] Above KSh1000

3d) According to you, can the LAMP method be applicable in a health facility for malaria diagnosis?

Yes [ ] No [ ]

3e) If LAMP method was to be adopted in the healthcare system, would you use it for malaria diagnosis?

Yes [ ] No [ ]

4a) If LAMP is adopted in the healthcare system, what would be the advantages?

[ ] High sensitivity

[ ] High specificity

[ ] Cheap in terms of cost compared to PCR

[ ] Easy to perform

[ ] Good turnaround time

[ ] None

[ ] Other (Specify)

_____

4b) If LAMP is adopted in the healthcare system, what would be the disadvantages?

[ ] Requires an equipment

[ ] Requires trained personnel

[ ] Long turnaround time

[ ] None

[ ] Other (Specify)

_____

5a) How can you rate the LAMP assay?

1 [ ] 2 [ ] 3 [ ] 4 [ ] 5 [ ]

KEY: 1= Very Poor, 2= Poor, 3= Good, 4= Very Good, 5= Excellent

5b) How can the LAMP assay be made better for malaria diagnosis?

_____

_____

_____

Appendix VI: Study Participants Perception of Malachite Green Loop-Mediated Isothermal Amplification (MG-LAMP) Assay

INSTRUCTIONS: ***Please, Tick Where Applicable***

Name of the Health Facility_____

Name/Code of the Interviewer_____

Patient Code/ID_____ Age_____

Gender: Male [ ]

Female [ ]

Residence_____

1a) What is your level of education?

[ ] Some primary

[ ] Complete primary

[ ] Some secondary

[ ] Complete secondary

[ ] Post-secondary education (certificate, diploma, graduate)

1b) Why did you visit the health facility today?

[ ] Sick

[ ] Accompanied a friend

[ ] Medical check-up

[ ] Told about the LAMP study

[ ] Other (Specify)

_____

_____

1c) Did you suspect what you were sick of?

Yes [ ] No [ ]

1d) What were the signs and symptoms that you had?

[ ] Fever

[ ] Headache

[ ] Joint pains

[ ] Vomiting

[ ] Tiredness

[ ] Other (Specify)

_____

_____

1e) What did the healthcare provider do?

[ ] Measured body temperature

[ ] Measured body weight and blood pressure

[ ] Prescribed medication

[ ] Requested a malaria test

[ ] Other (Specify)

_____

_____

1f) If it was malaria test, which type of test was carried out for diagnosis?

[ ] Microscopy

[ ] Rapid diagnostic test (RDT)

[ ] Other (Specify)

_____

_____

1g) Where was the test done?

[ ] Laboratory

[ ] Clinician's office

[ ] Nurse Station

[ ] Ward

[ ] Other (Specify) _____

_____

1h) How much did you pay for malaria test?

[ ] Less than KSh100

[ ] KSh100-KSh200

[ ] More than KSh200

1i) How long did you wait for the results?

[ ] Less than 30minutes

[ ] 30minutes to 60minutes

[ ] More than 60minutes

NOTE: Briefly explain to the participant about LAMP.

Malachite-Green Loop Mediated Isothermal Amplification Assay

Introduction

Loop-mediated isothermal amplification (LAMP) is an isothermal DNA amplification method which was developed in 1998 by Eiken Chemical Company, Japan. LAMP requires four to six different primers (F1P, B1P, F3, B3, LPF, and LPB), that are designed specifically to recognize six to eight specific gene sequences. DNA amplification is accomplished with the use of DNA polymerase with strand-displacing activity. This strand-displacing activity allows amplification under isothermal conditions in contrast to polymerase chain reaction (PCR) where a thermal denaturation step is essential.

Procedure

Fifty microliters of the collected blood will be used to extract DNA for the RealAmp assay. DNA will be released from the whole blood by boiling the blood in a heat-block at 95°C for 10 minutes. The tubes will be spun down using a centrifuge for 5 minutes and the supernatant (containing the DNA) will be collected and used in the LAMP assay. Five microliters of this supernatant will be used in the LAMP assay. The Malachite Green (MG) malaria LAMP assay will be performed in a 20µL total reaction volume, 0.004% MG, 8 units of Bst polymerase and 5µL of template DNA. Mitochondria Plasmodium genus-specific primers will be used to amplify the DNA. The amplification will be carried out at 63°C for 60 minutes using a simple heat- block. Samples will then be allowed to cool for 15 minutes before being scored by three independent human readers. Positive samples retain a light green/blue malachite green color while negative samples turn colorless.

2a) According to the explanation which you have been given about LAMP method for malaria diagnosis, can it be applicable in a health facility?

Yes [ ] No [ ]

2b) What is your opinion about the turnaround time for LAMP method for malaria diagnosis?

[ ] Long

[ ] Short

[ ] Satisfactory

[ ] Not applicable

2c) How much are you willing to pay for the LAMP method for malaria diagnosis?

[ ] Less than KSh100

[ ] KSh100 to KSh500

[ ] KSh500 to KSh1000

[ ] Above KSh1000

2d) How long are you willing to wait for the results of the LAMP method for malaria diagnosis?

[ ] Less than 30minutes

[ ] 30minutes to 60minutes

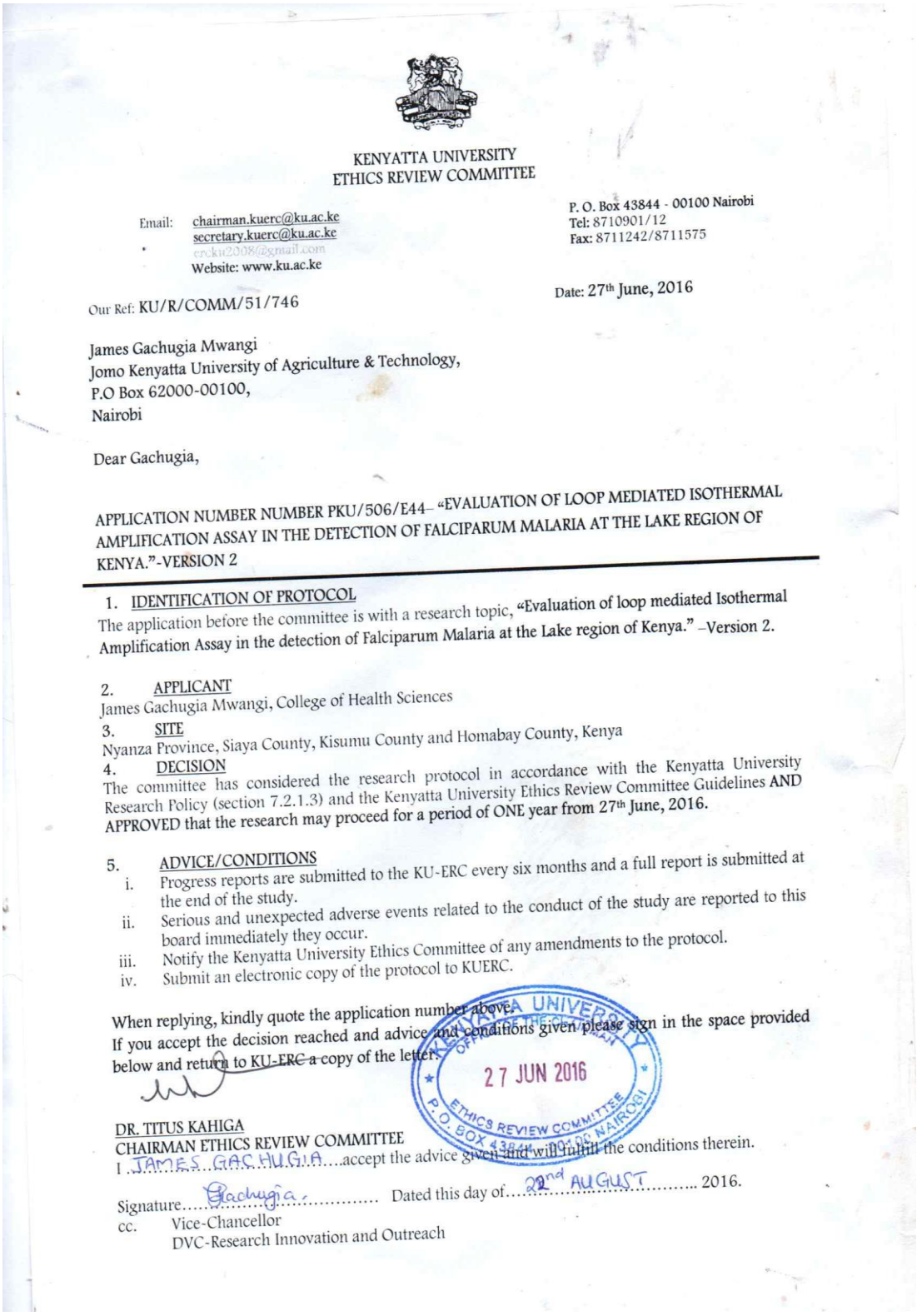
[ ] More than 60minutes

3a) How can you rate the LAMP method?

1 [ ] 2 [ ] 3 [ ] 4 [ ] 5 [ ]

KEY: 1= Very Poor, 2= Poor, 3= Good, 4= Very Good, 5= Excellent

Appendix VII: Ethical Approval



KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

Email: chairman.kuerc@ku.ac.ke
secretary.kuerc@ku.ac.ke
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P. O. Box 43844 - 00100 Nairobi
Tel: 8710901/12
Fax: 8711242/8711575

Our Ref: KU/R/COMM/51/746

Date: 27th June, 2016

James Gachugia Mwangi
Jomo Kenyatta University of Agriculture & Technology,
P.O Box 62000-00100,
Nairobi

Dear Gachugia,

APPLICATION NUMBER NUMBER PKU/506/E44– "EVALUATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION ASSAY IN THE DETECTION OF FALCIPARUM MALARIA AT THE LAKE REGION OF KENYA."-VERSION 2

1. IDENTIFICATION OF PROTOCOL
The application before the committee is with a research topic, "Evaluation of loop mediated Isothermal Amplification Assay in the detection of Falciparum Malaria at the Lake region of Kenya." –Version 2.

2. APPLICANT
James Gachugia Mwangi, College of Health Sciences

3. SITE
Nyanza Province, Siaya County, Kisumu County and Homabay County, Kenya

4. DECISION
The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines AND APPROVED that the research may proceed for a period of ONE year from 27th June, 2016.

5. ADVICE/CONDITIONS
i. Progress reports are submitted to the KU-ERC every six months and a full report is submitted at the end of the study.
ii. Serious and unexpected adverse events related to the conduct of the study are reported to this board immediately they occur.
iii. Notify the Kenyatta University Ethics Committee of any amendments to the protocol.
iv. Submit an electronic copy of the protocol to KUERC.

When replying, kindly quote the application number above.
If you accept the decision reached and advice and conditions given please sign in the space provided below and return to KU-ERC a copy of the letter.

KENYATTA UNIVERSITY OFFICE OF THE CHAIRMAN 27 JUN 2016 ETHICS REVIEW COMMITTEE P.O. BOX 43844 - 00100 NAIROBI

DR. TITUS KAHIGA
CHAIRMAN ETHICS REVIEW COMMITTEE

I JAMES GACHUGIA ....accept the advice given and will fulfill the conditions therein.

Signature Gachugia Dated this day of 2nd AUGUST 2016.

cc. Vice-Chancellor
DVC-Research Innovation and Outreach