MOLECULAR DETECTION OF HUMAN MALARIA PARASITES USING HIGH RESOLUTION MELTING ANALYSIS IN MOROGORO MUNICIPALITY, TANZANIA

REHEMA M. MAKOY

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

Microscopy and rapid diagnostic tests (RDTs) are common tools for diagnosing malaria, but are deficient in detecting low *Plasmodium* parasitaemia and mixed infections. Molecular detection has overcome the limitations by providing greater sensitivity and specificity in *Plasmodium* species detection. This study was conducted in Morogoro region employing a new molecular diagnostic tool that combines the high sensitivity and specificity of nested PCR (nPCR) and high resolution melting analysis (HRMA). Samples were collected from 301 malaria suspected patients from SUA Health Centre between August-October, 2018 and Sabasaba Health Centre between October-December, 2018. Samples were screened for malaria parasites by microscopy at SUA and mRDTs at Sabasaba, and later analysed by nPCR-HRMA for *P. falciparum, P. malariae and P. ovale*. Data was analysed using EPI info version 7 and IBM SPSS version 20 software. Sensitivity and specificity of the three diagnostic methods were assessed, variables calculated with 95% confidence interval (CI). Microscopy had a lower sensitivity in diagnosing malaria compared to nPCR-HRMA ($p \leq 0.0361$). Among all samples which tested malaria positive by microscopy, only 68.75% were positive by nPCR-HRMA and the rest negative. Similarly, 6.45% samples which were initially found to be malaria negative by microscopy were positive by nPCR-HRMA. Most of the results from mRDTs were in agreement with nPCR-HRMA results ($p \leq 0.2987$). Among the samples which tested positive by mRDT, 83% were also positive by nPCR-HRMA while only 1.9% of the samples which had tested negative by mRDT were positive by nPCR-HRMA. In addition nPCR-HRMA provided accurate diagnosis of infecting

single or mixed *Plasmodium* species in samples that were missed by mRDT and microscopy. Deployment of improved malaria diagnostic methods like the nPCR-HRMA might help in improvement and better understanding of the epidemiology of *Plasmodium* species, with a particular focus on identifying asymptomatic carriers and for designing appropriate treatment.

DECLARATION

I, Rehema M Makoy, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

…… ……

Rehema M. Makoy Date

(MSc. Candidate)

The above declaration confirmed

…….. ……

Prof. Benezeth M. Mutayoba Date

(Supervisor)

……. ……..

Prof. Allen Lewis Malisa Date

(Supervisor)

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DEDICATION

I dedicate this thesis to my beloved family and friends, who have meant and continue to mean so much to me.

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LIST OF ABBREVIATIONS AND SYMBOLS

µL	Microlitre
CDC	Centre for Diseases Control
CI	Confidence Interval
DBS	Dry blood spot on filter paper
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
GC	Guanine-Cytosine
HRM	High Resolution Melting
HRMA	High Resolution Melting Analysis
HRP	Histidine Rich Proteins
IHRDC	Ifakara Health Research and Development Centre
ITNs	Insecticide Treated Nets
LoD	Limit of Detection
mL	Millilitre
MRCC	Medical Research Coordinating Committee
mRDTs	Malaria Rapid Diagnostic Tests
NIMR	National Institute for Medical Research
nPCR	Nested Polymerase Chain Reaction
ORs	Odd ratios
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Principal Investigator

pLDH	Plasmodium lactate dehydrogenase
qPCR	Qualitative Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic acid
RDT	Rapid Diagnostic Test
rRNA	Ribosomal Ribonucleic acid
SUA	Sokoine University Agriculture
Tm	Melting temperature
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes. Despite being preventable and curable, malaria remains a global disease affecting millions of people in tropical and sub-tropical regions. The World Health Organization (WHO) estimates 300 to 500 million cases of malaria infections resulting in over one million deaths globally each year (Mangold *et al.,* 2005). It is further estimated that 40% of the world's population mostly those living in the poorest countries are at risk for malaria. Existing records show that high malaria risk areas include large portion of central and South America, Africa, the Indian subcontinent, South East Asia and the Middle East (CDC, 2018). Ninety percent of malaria cases occur in Sub-Saharan Africa where the disease is endemic, with most cases occurring in children under five years of age. The high costs of malaria treatment continue to trap families in a cycle of illness, suffering and poverty (WHO, 1999)**.**

Tanzania has the third largest population at risk of malaria in Africa; over ninety percent of people live in areas where malaria is endemic (Mathania *et al.,* 2016). In this country, ten to twelve million people contract malaria and 60,000 to 80,000 die from the disease per year, most of them being children. The data provided by the Tanzania National Bureau of Statistics in 2017, however show that the overall malaria incidences in Tanzania have generally dropped significantly from 14.4% (2015) to 7.3% (2017) partly due to maternal education, household wealth and the

national efforts of encouraging people to use insecticide treated bed nets (ITNs) for protection against Anopheles mosquitoes (Mathania *et al.*, 2016). Few regions which still show higher malaria incidences include Kigoma (24.4%), Geita (17.3%), Kagera (15.4%), Mtwara (14.8%), Ruvuma (11.8%), Lindi (11.7%), Tabora (11.7%), Mara (11.2%), Morogoro (9.5%) and Mwanza (8.9%) (URT, 2018).

Human malaria infections exhibit a broad clinical spectrum ranging from asymptomatic infection to severe life-threatening disease. Globally five *Plasmodium* species are known to cause malaria in humans: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. Accurate differentiation of infecting *Plasmodium* species is essential for selecting proper treatment (Mangold *et al.*, 2005). This is more important when differentiating *P. falciparum* from the others, since this species is responsible for approximately 95% of the deaths due to malaria (Stoppacher *et al.*, 2003).

Prompt and accurate diagnosis being an important strategy in the fight against malaria, universal access to parasitological test is part of the WHO objectives (WHO, 2014). Microscopic examination based on Giemsa-stained thick and thin blood smears is considered the reference gold standard for malaria diagnosis; and still remains the most common malaria diagnostic tool, due to its inexpensiveness and simplicity (Wongsrichanalai *et al.*, 2007). However the microscopy technique is not only time-consuming to prepare, read, and interpret the slides, but also fail to detect mixed infections, when one of the *Plasmodium* species is present at low levels (<100 parasites/mL), or modified by anti-malarial drug treatment (Mangold *et al*., 2005). Previous studies have shown that even with experienced microscopists, misdiagnosis

occurs, particularly in cases of mixed infection or low parasitaemia (Hanscheid, 2003).

Available complementary method includes Rapid Diagnostic Test (RDT); these are alternative assays based upon commercially developed antigen-capture test kits that have been designed primarily to diagnose *P. falciparum*. The test is quick and easy to perform. However, limitations of the antigen-capture test encountered include its inability to assess level of parasitaemia in infected patients as well as failure to detect *P. vivax, P. malariae* or *P. ovale* specifically. Additionally, RDT specifically the Histidine Rich Protein (HRP-2) is frequently less sensitive than microscopy, and can produce false positive results through detection of persistent antigenemia following parasite clearance and treatment.

Molecular based methods such as conventional Polymerase Chain Reaction (PCR) are also used in malaria diagnosis. These methods are highly sensitive and rapid Shakely *et al.* (2013), they be can be performed on hundreds of samples at relatively short time (Zimmerman, 2004). Different studies done in various countries have showed that conventional nested PCR techniques can detect parasites at lower concentration and mixed infections, as well as identify all *Plasmodium* species in single or mixed infection Snounou *et al*. (1993) by targeting the mitochondrial genome. An advantage of using this target is that mature gametocytes have four to eight mitochondrial organelles per parasite and each organelle can have about twenty copies of the mitochondrial genome (Mbiri *et al.,* 2017). This potentially makes the amplification of the genome target by PCR very sensitive, even if the parasite level in the blood of patients is very low (Isozumi *et al.,* 2015).

HRMA is a highly sensitive molecular technique which is able to identify smaller differences in PCR amplicons down to the single base level by comparing different melting curves which are produced by alleles (Smith *et al*., 2009). It is therefore perfect for single nucleotide polymorphism genotyping, species identification, sequence matching and mutation scanning without the need for any further separation and additional processing after PCR (Erali *et al*., 2008). HRMA as the name suggests analyses the melting curves over extremely small ranges and can distinguish between different genotype based on their variations. It is possible to detect small differences based on very small changes in the melting curve due to high resolution offered by this technology. This study was done for the first time in Morogoro region of Tanzania where a newly developed molecular technique involving nested PCR and High Resolution Melting Analysis was used in malaria diagnosis.

1.2 Problem statement and justification

In malaria endemic countries like Tanzania, individuals are often found to display simultaneous infections involving more than one malaria species. These mixed infections can produce nonspecific clinical manifestations of malaria, which can contribute to presumptive diagnosis and treatment (Zimmerman, 2004). They are underreported because of limitations in detection methods currently being used including microscopic and/or RDT-based methods. The existing microscopic and RDT-based methods also suffer from low sensitivity detecting infections of 100 parasites /µL and 50-400 parasites /µL respectively and below (Chua *et al.*, 2015).

For this reason, efforts have been put in developing molecular based laboratory tools that are capable of providing accurate diagnosis of all human malaria species concurrently (Kipanga *et al.*, 2014). These are essential for estimating clinical prognosis and monitoring therapeutic responses, particularly within geographical areas that harbour drug-resistant parasites. Molecular methods such as non- multiplex (nested PCR, and real-time PCR) and multiplex PCR (Rubio, 1999), which allow accurate species identification and are valuable for distinguishing species in cases of mixed malaria infection have been described (Snounou *et al.*, 1993). These include semi-nested multiplex described by Rubio (1999) and single-round multiplex PCR, a high-throughput multiplex nuclease quantitative PCR (qPCR) by Reller *et al.* (2013), real-time multiplex polymerase chain reaction (PCR), merozoite surface antigen gene (MSP) multiplex PCR, and the PlasmoNex Multiplex PCR Kit (Lau *et al.*, 2015). Although these methods are very sensitive and widely used they are however, expensive and laborious for routine use.

Morogoro is among the regions with high prevalence of malaria and the common diagnostic methods currently used in almost all health centres depends on either microscopy and/or RDT based approach which are generally less sensitive for cases of very low parasitaemic infections and/or asymptomatic malaria. Similar diagnostic methods continue to be used in Tanzania at a nationwide scale and uses of highly sensitive molecular based methods are still rare (Shakely *et al.*, 2013).

An alternative molecular method that is rapid, cheaper and highly sensitive capable of accurately diagnosing all human malaria species concurrently, and based on nested PCR High Resolution Melting Analysis (nPCR-HRMA) approach has been

described in Kenya (Kipanga *et al.*, 2014). This method is powerful and can detect rapidly parasitaemia with greater sensitivity (LoD = 236 parasites/mL) highly suitable for differentiating very low parasitaemia infections in asymptomatic patients. The present study aimed at assessing the suitability of using (PCR-HRMA) tool for the first time in Tanzania for malaria diagnosis in selected health centres in Morogoro Region.

1.3 Hypotheses

- HRMA has a high sensitivity compared to microscopy in detecting different malaria parasite species.
- HRMA has a high sensitivity compared to mRDTs in detecting different malaria parasite species.

1.4 Objectives

1.4.1 General objective

To perform molecular diagnosis of human *Plasmodium* species infections using a high throughput PCR-HRMA in Morogoro.

1.4.2 Specific objectives

i. To standardize and validate the PCR-HRMA tool for use in malarial diagnosis

ii. To assess the suitability of HRMA for accurate identification of *plasmodium* species in single or mixed infections among febrile patients.

iii. To compare the relative sensitivity and accuracy of PCR-HRMA, microscopy and mRDT in the screening of blood samples from febrile patients.

CHAPTER TWO

2.0 Literature review

2.1 Overview

Malaria is a mosquito-borne infectious disease affecting humans and other animals caused by parasitic protozoan belonging to the genus *Plasmodium*. Although novel exceptions have been reported, it is commonly agreed that *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale* are the four species that cause human malaria (Tahar *et al.*, 2002). It has been reported that the most severe form of malaria is caused by *P. falciparum* which causes variable clinical features including fever, chills, headache, muscular aching and weakness, vomiting, cough, diarrhea and abdominal pain. Other symptoms related to organ failure may supervene, such as acute renal failure, pulmonary edema, generalized convulsions, circulatory collapse, followed by coma and death (WHO, 2019).

Malaria parasite is transmitted by female anopheles mosquitoes which bite mainly between dusk and dawn; transmission occurs more during wet season than dry season. The biggest risk factor for developing malaria is to live in or visit areas where the disease is common. Disease severity is mostly influenced by interactions between parasite, human host and environmental factors (Kateera *et al.*, 2016). Young children, pregnant women, people who are immunosuppressed and elderly travelers are particularly at risk of severe disease. Malaria in non-immune pregnant travelers increases the risk of maternal death, miscarriage, stillbirth and neonatal death.

2.2 Mixed Infections

The coexistence of more than one parasite species in the same individual may be mediated by host and pathogen factors, such as the host immune response initially directed against the species or genotype at the highest density, thereby favoring the persistence of infection at lower density in a particular host (Bruce & Day, 2002). The species/genotype coexistence model is controlled by parasite density-dependent regulation mechanisms; this model suggests that parasitaemia of the first infecting species (which has the highest prevalence amongst the target population) is down regulated on co infection with the second species (which has the lowest prevalence) (Bruce *et al.,* 2011). However, when the most prevalent species exceeds a threshold, the hosts' immune response is triggered to limit the infection; such a mechanism is turned off once the parasite density is under control, thereby favoring population growth of the second species in mixed infections and persistence of the parasites in the host

Mixed-species malaria infections are often unrecognized or underestimated (Kim *et al.,* 2019). In malaria endemic regions, it is not uncommon to find humans harboring mixed infections, often complicating routine microscopic diagnostic methods calling for provision of accurate treatment interventions of such patients (Golassa *et al.,* 2013). Studies suggest that mixed infections have been discussed since 1930, when Knowles and White acknowledged difficulties that microscopists might encounter in accurately documenting their findings from examinations of blood smear (Knowles & White, 1930). Antimalarial treatment studies have also contributed insight regarding mixed *Plasmodium* species infections by revealing undocumented

infection of a second species following successful treatment of first specie (Zimmerman, 2004).

When humans harbor multiple *Plasmodium* species, the varying patterns in species-specific parasitaemia and mixed-species prevalence characterize malaria in different endemic regions. If mixed-species malaria is misdiagnosed as a single *P. vivax* infection, treatment of *P. vivax* increases *P. falciparum* parasitaemia (Tajebe *et al.*, 2014). Mixed-species infections will increase the possibility of anti-malarial drug resistance, and then a drug-resistant population of *Plasmodium* parasites will emerge (Lee *et al.*, 2011).

To facilitate proper management of malarial cases, it is prudently necessary to ensure that accurate species identification and correct prognosis based on appropriate differential diagnosis is done for therapeutic decisions and proper prescription of right medication. This unequivocally also helps in tracking malaria elimination efforts since a delay or failure in detecting *P. falciparum* leads to infection aggravation and rise of the mortality rate, particularly in low or non-immune people (Lau *et al.*, 2015). Mistreatment of a single or multiple species will have serious clinical consequences therefore for more precise epidemiological decisions, an accurate identification of low parasitaemia and mixed-species infections are necessary.

2.3 Malaria diagnosis

2.3.1 Microscopic tests

Microscopic test involves staining and direct visualization or examination of the red blood cells for intracellular malaria parasite under the microscope (WHO, 2009). The

patient's blood specimen is spread as a thick or thin blood smear and stained with Romanowsky (Giemsa). It is relatively simple and the method is capable of showing the magnitude of parasitaemia. This diagnostic method is the most widely utilized approach for generating malaria infection data for epidemiological studies focused on mixed-species infections (Wongsrichanalai *et al.,* 2007). One of the disadvantages of the blood-smear microscopy techniques is the misdiagnosis of *Plasmodium* species. Often the method fails to detect mixed infections, when one of the *Plasmodium* species is present at low levels (<100 parasites/mL), or modified by anti-malarial drug treatment (Scopel *et al.,* 2004). The precision of blood-smear diagnostic approach is also compromised by the quality of the blood slide preparation, the number of microscope fields analyzed (blood volume), and the microscopists' expertise and experience (Doni *et al.,* 2016).

Microscopic observation of *P. falciparum* infection is mostly influenced by its parasite density. Parasite density of infections by other species is usually low compared to *P. falciparum* (Tajebe *et al.,* 2014). Therefore, other *Plasmodium* species are easily missed, particularly in the absence of symptoms. Moreover, in mixed infections, the background of large numbers of *P. falciparum* parasites makes the observation difficult to differentiate other species.

2.3.2 Rapid Diagnostic Tests

In recent years, the development of Rapid Diagnostic Test (RDT) based methodologies introduced new and better strategies for malaria diagnosis (Zimmerman, 2004) and in Tanzania, the use of RDT was officially adopted in 2011 as a complementary rapid tool to microscopy in routine malaria diagnosis at all

health Centers in the country (Adinan, 2015). Rapid diagnostic test (RDT) is a device that detects malaria antigen by immunochromatographic assay with monoclonal antibodies directed against the target parasite antigen and impregnated on a test strip (Wongsrichanalai *et al.,* 2007). Usually the result is obtained in 5-20 minutes as a colored test line, being simple to perform and interpret; its consumption has increased in the developing countries.

The method detects specific antigens (proteins) produced by malaria parasites in the blood of an infected individual. Target antigens include; histidine-rich protein 2 (HRP-2) which can be specific to *P. falciparum* or *P. vivax* (Shiff *et al*., 1993), the second is Plasmodium lactate dehydrogenase (pLDH) which can be specific to *P. falciparum* or *P. vivax* or be a variant that is common to all *Plasmodium* species (pan specific) and the last is *Plasmodium* aldolase, which is pan specific (Palmer *et al.,* 1998). The histidine-rich protein 2 (HRP-2) is a histidine and alanine rich, water-soluble protein, which is localized in several cell compartments including the parasite cytoplasm. The antigen is expressed only by *P. falciparum* trophozoites (Iqbal *et al.,* 2000).

These assays are quick and easy to perform. However, their major limitation is that they require the use of a very small amount of blood, usually 5–15 µL, which can provide a misdiagnosis in patients with very low parasitaemia. Also RDTs are generally specific for detecting *P. falciparum* and *P. vivax* infections, but are non-specific for other malaria species (Shokoples *et al.,* 2009). Additionally, RDTs are known to produce false positive results because of residual antigen, which can persist

for weeks after treatment and parasite clearance (Mixson-Hayden *et al.*, 2010), they also fail to determine the magnitude of parasitaemia.

2.3.3 Molecular based techniques

Since the late 1980s, polymerase chain reaction (PCR)-based methods have been applied in malaria diagnosis due to their high sensitivity, rapidity and reproducibility (Wongsrichanalai *et al.,* 2007). A number of nested and semi-nested PCR methods have been developed over the years (Pinheirob *et al.,* 1993), which allow for detection of up to four human *Plasmodium* species (*P. falciparum, P. vivax, P. malariae and P. ovale*) concurrently. These molecular methods have been used successfully in various epidemiological surveillance studies of malaria cases in numerous countries (Reller *et al.,* 2013; Shakely *et al.,* 2013; Kipanga *et al.,* 2014; Doni *et al.,* 2016). Snounou and others developed a nested PCR method, which targets the 18S ribosomal DNA (rDNA) gene and allows for the discrimination between distinct malaria species based on differentially sized PCR products (Snounou *et al.,* 1993). This conventional nested PCR techniques offers the capacity detecting parasites at lower concentration of about 5 parasites/μL (Harris *et al.,* 2010) and mixed infections, as well as discrimination of the Plasmodium infecting species (Golassa *et al.,* 2013). Efforts are still underway to develop single-reaction assays for the rapid and specific identification of all five human malaria species. Molecular techniques are generally superior to antigen-detection tests and microscopy for detecting pathogens in biological samples (Okell *et al.,* 2009; Shakely *et al.,* 2013).

Recently new novel malaria molecular diagnostic method has been described which is faster, cheaper and robust with high sensitivity and accuracy in detecting single or mixed infections and low *Plasmodium* parasitaemia infections in febrile patients. This approach combines the power of nested PCR with High Resolution Melting Analysis (nPCR-HRMA) to detect and differentiate various *Plasmodium* species in malaria patients. HRM analysis is a post-PCR process, first the DNA involve amplification of the region of interest in the presence of a specialized double stranded DNA (dsDNA) binding dye and gradual denaturation of amplicons by increasing the temperature in small increments in order producing a characteristic melting profile that is called melting analysis (Chua *et al.,* 2015).

The intercalating dyes such as Evagreen and Syto 9 are incorporated into the dsDNA, the dyes fluorescence brightly when incorporated in dsDNA and undergo solvent quenching as the dsDNA is melted by gradual increase in temperature, hence fluorescence poorly when that dsDNA slowly melts into ssDNA releasing the dye. After the melting procedure, a "melt curve" is obtained by plotting fluorescence intensity as a function of temperature. The melt curve profile is unique for a particular DNA sequence hence by analyzing the melt curve profile one can identify variations in a DNA sequence. Species are identified and discriminated based on their differences in complementarities, GC content, DNA sequence and sequence length. Usually this is virtually done by comparing unknown melt profiles to known control melt profiles and by software based automatic genotyping. This technique works reliably with PCR products not longer than 400 base pairs, the shorter the amplicons the better. (Ouso *et al*., 2019)

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design

This study involved an evaluative research design whereby blood samples from febrile patients were analysed for presence of malarial parasites by HRMA and the results were compared to the microscopy and mRDT results.

3.2 Study area description

The study was conducted in Morogoro Urban District, which lies at the base of the Uluguru Mountains. The district is located at an altitude of 500–600 m above sea level and between longitude 37º–39ºE and latitude 6º–5ºS. It enjoys a mixture of warm and cool temperatures ranging between 27 and 33.7 °C in the dry/warm season and 14.2 and 21.7 °C in cold/wet season. The district being located on the lower slopes of Uluguru mountains experiences heavy rainfall from February to June creating seasonal mosquito breeding sites due to the areas being flat with high risk of standing water. Since the climate is favourable, inhabitants of the area depend on agriculture as their main economic activity.

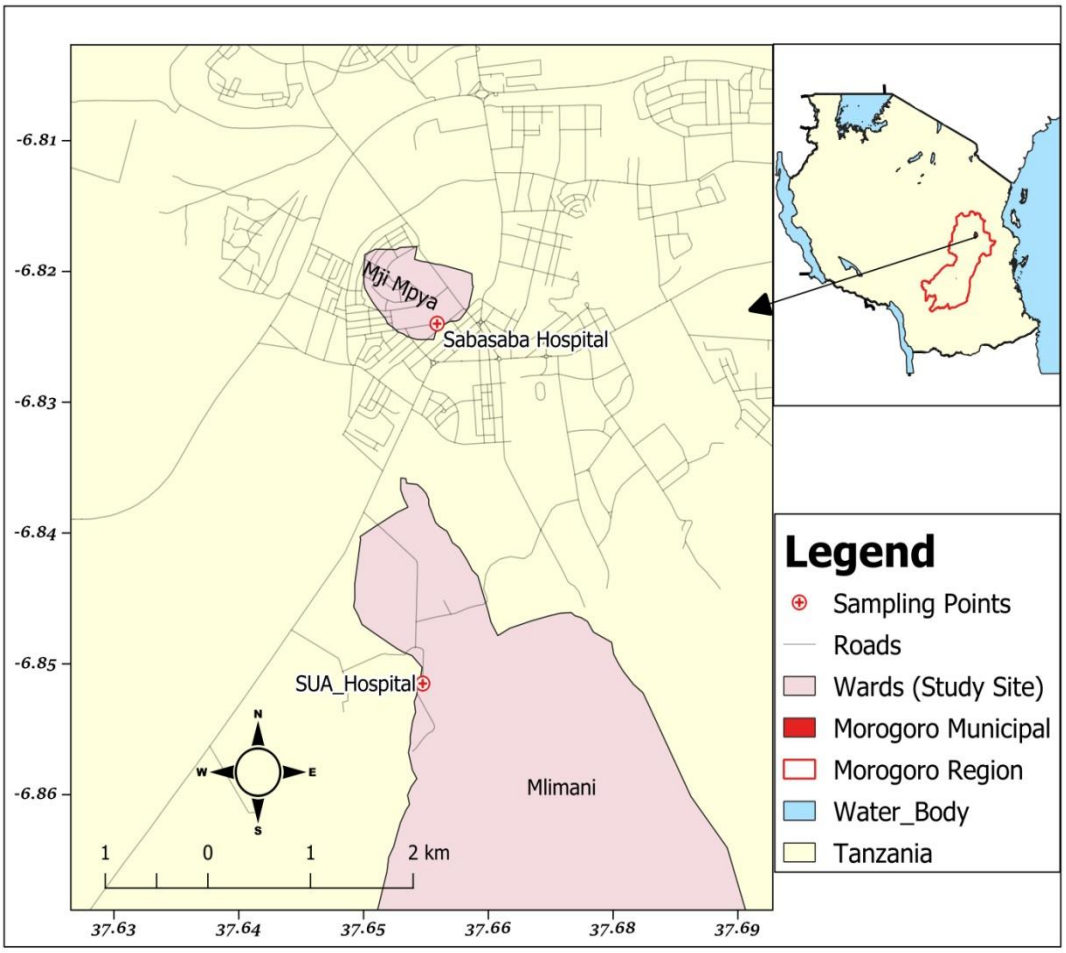

Figure 1: Map showing geographical location of study areas

3.3 Study population

The study involved all patients without age restriction visiting two health centres namely Sabasaba Health Centre managed by the Morogoro Municipal Council, Tanzania and SUA Hospital managed by the Department of Health Services and located at SUA main campus.

The Sabasaba Health Centre is centrally located in Morogoro Municipality and do receive wide variety of patients across the region, while SUA Hospital is mainly

involved in handling patients coming within and few from outside the University. Patients presenting with febrile conditions visiting the two health centres were all recruited in the study. Sabasaba Health Centre mainly relies on mRDTs in malaria diagnosis while SUA Hospital relies heavily on conventional microscopy malaria diagnostic approach.

3.3.1 Patient Inclusion criteria

- Individuals presenting with febrile symptoms visiting health centers seeking health care
- Individuals who willingly consent to participate in the study
- Both male and female, at all ages were involved in the study

3.3.2 Exclusion Criteria

- Individuals unwilling to take part in the study

3.4 Sampling size

According to the data provided by the Tanzania National bureau of statistics, the prevalence of malaria in Morogoro region is estimated to be 9.5%.

Thus the sample size was calculated from the following formula which was adopted from Singh & Masuku (2014) $N = \frac{Z^2 P(1-P)}{d^2}$

Where: N= the total sample size in the sample

z = a standardized normal deviate value that correspond to a level of statistical significance of $P \leq 0.05$ which is 1.96

p = estimate of proportion or prevalence (9.5%)

d = margin of error on p which is 0.05

$\frac{1.96^2 0.095\ (1-0.095)}{0.05^2}$, a total of 301 people were recruited in the study, 140 from SUA Hospital and 161 from Sabasaba Health Centre

3.5 Study procedures and Laboratory analyses

3.5.1 Blood sample collection

Blood was collected from patients visiting the two health centres presenting with febrile symptoms; fever above 37.5°C, headache, joint pains, diarrhoea, cough, abdominal pain, constipation, nausea and any other related symptoms. A finger prick slide and filter paper bloodspot was collected from each individual by the respective hospital health attendant. Bloodspot on slides were first screened for the presence of *Plasmodium* parasites using microscopy at SUA and rapid diagnostic test (RDT) at Sabasaba and the results were recorded. The Whatmann filter paper bloodspot samples were air-dried and stored at room temperature in self-sealing khaki envelopes, then transported to the laboratory at Sokoine University of Agriculture (SUA) for further analyses. About 140 of the subjects were recruited from SUA Health Centre between 9th August, 2018 and 11th October, 2018 and the rest 161 from Sabasaba Health Centre between 15th October, 2018 and 13th December, 2018.

Dried standard bloodspot for known species (*P. falciparum, P. malariae* and *P. ovale*) were obtained from the National Institute for Medical Research (NIMR) laboratory in Tanga. The DNA obtained from these standard samples was used as references to identify all *Plasmodium* species found in unknown samples collected in the study.

3.5.2 Extraction of total DNA from blood

A sector of the dried bloodspot on the 3MM Whatmann filter paper was excised using a sterile blade and soaked in a 1ml of 0.5% saponin in 1x phosphate buffered saline (PBS) overnight, to enable the haemoglobin be released into the wash leaving parasite DNA on the paper. The PBS used was made by dissolving 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8mM KH_2PO_4 in distilled water adjusted at pH 7.4. The sector was then washed twice in 1ml 1x PBS, each time supernatant removed after vortexing and centrifuging at 10,000 RCF for 1 minute. Finally the pellets were boiled at 90^0C for 15 min in 100 µL PCR quality water containing 50 µL 20% Chelex 100 (Sigma-Aldrich) suspension pH 9.5 at 10,000g RCF for 5 minutes and the supernatant was then stored at -20^0C until required (Malisa *et al.,* 2010). The DNA concentration and quality of each extract was measured by using a nanodrop spectrophotometer (code number 2894312, serial number. 120367 BIOCH ROM LTD, CAMBRIDGE ENGLAND).

3.5.3 Detection of Plasmodium parasites

The extracted DNA was amplified using nested PCR targeting 18s rRNA genes in *Plasmodium* DNA (Kipanga *et al.,* 2014). The 18s rRNA was selected as the target since it contains both highly conserved and variable regions and at least five copies of the gene are dispersed on separate chromosomes of the *Plasmodium* genome (McCutchan, 1995).

Two sets of primers were used; the first pair used for the primary amplification was PL-1459-Forward: CTG GTT AAT TCC GAT AAC and PL-1706-Reverse: TAA ACT TCC TTG TGT TAG AC. The second pair used for the nested amplification

reaction was PL-1473-Forward (3'-TAA CGA ACG AGA TCT TAA-5') and PL-1679-Reverse (3'-GTT CCT CTA AGA AGC TTT-5').

The DNA extract was diluted five (5) times and used as template for the primary amplification reaction while ten-fold dilutions of the primary PCR products were used as templates for the secondary amplification reaction. Each of the two amplification reactions were carried out in 10 µL final reaction volumes consisting of 2 µL DNA template, 3 µL Hot Firepol® HRM mix kit (Solis BioDyne, Estonia), 0.5 µL of 0.5 µM of both primers and 4 µL nuclease free PCR water.

The PCR conditions for the primary and secondary amplifications as well as HRM Analysis were adopted from Kipanga *et al*. (2014) with modifications. The touchdown PCR thermal conditions consisted of an initial denaturation at 95°C for 5 minutes, 45 cycles of denaturation at 94°C for 20 seconds, decreasing annealing temperatures from 66°C to 54°C for 30 seconds (cycles 1–5), 60°C for 40 seconds (cycles 6–10), 54°C for 50 seconds (cycles 11–45), and extension at 72°C for 30 seconds. A final extension of 72°C for 3 minutes was included before HRM analysis. Upon completion, the nested PCR process was transitioned into the melting phase (HRM) in the same closed tube system yielding distinct melting profiles. The PCR-HRM was performed using a Magnetic Induction Cycler (MIC) (Bio Molecular Systems, Australia) which can run a maximum of up to 48 samples per run in 2 hr period. The set of conditions for HRM included 0.2°C incremental temperature increases from 75°C to 90°C, with fluorescence acquisition at the end of each 2 second temperature increment.

3.6 Data management and analysis

Data entry and storage was done using Ms Excel followed by analysis using EPI info version 7 and IBM SPSS version 20 software. Frequencies and cross tabulation were calculated to obtain proportions for each of the study variables calculated with 95% confidence interval (CI) and corresponding p-values, as appropriate. The prevalence was assessed as the existing malaria positive cases diagnosed by each technique at a particular point. Prevalence of malaria infection detected by microscopy and mRDT was compared with the prevalence of infection determined by PCR-HRM. Sensitivity values for each method were calculated as the percentage of PCR-HRM positive and negative individuals, respectively, who were correctly identified as such by each method (this is the number of false-positive and negative results obtained by microscopy and mRDT, compared with PCR-HRM "reference standard" results).

3.7 Ethical Consideration

Ethical approval was obtained from Medical Research Coordinating committee of Tanzania's National Institute for Medical Research (NIMR/HQ/R.8a/Vol.1X/2898). Consent was obtained from all individuals or their guardians before collection of samples. To protect the identity of the participants, only personal information (gender, age, location) was used during the analysis and all participants were assured of anonymity.

CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of study subjects

A total 301 patients presenting with febrile illness comprising of 168 females and 133 males visiting SUA health centre and Sabasaba health centre in Morogoro, Tanzania were enrolled in the study (Table 1). Subjects were between 0 and 84 years of age but the highest (27.24%) proportions of these patients were aged 0-4 years old. About 46.5% (n=140) of the subjects were recruited from SUA health centre between 9th August, 2018 and 11th October, 2018 and the rest 53.5% (n=161) from Sabasaba health centre between 15th October, 2018 and 13th December, 2018. Among the 140 samples collected from SUA, 124 samples were malaria negative and 16 positive by Light Microscopy solely used at the Centre. Likewise out of 161 samples which were sampled at Sabasaba, 155 were confirmed malaria negative and 6 positive my mRDT solely used at the Centre.

Table 1: General characteristics of study subjects

Method used in Malaria Diagnosis		**Malaria status**		**Total**
		Negative	**Positive**	
Light microscopy (SUA)	F	70	7	**77**
	M	54	9	**63**
	Total	**124**	**16**	**140**
mRDT (Sabasaba)	F	87	4	**91**
	M	68	2	**70**
	Total	**155**	**6**	**161**

4.2 HRMA profiles standardization

In order to use HRM capacity to correctly assign the *Plasmodium* species from samples obtained from SUA and Sabasaba health Centres, HRM protocol standardization was initially performed using samples provided by NIMR and known to harbour *Plasmodium* species of known identity. The Melt and HRM profiles generated from these samples were used as reference profiles. Melt analysis allowed the determination of peak dissociation temperature Tm of each sample from the melt data and was used as a measure of analytical specificity for each run. While High Resolution Melting (HRM) analysis allowed identification of DNA sequence variants including single base changes, incision, deletion and base pair substitution by analysing DNA melt curves. The analysis provided the capacity to analyse DNA samples according to their dissociation behaviour as the transition from dsDNA to ssDNA with increasing temperature. Melt curve analysis permitted the clear identification of each *Plasmodium* species control. Prevalence estimates of *Plasmodium* parasitaemia generated by each method were used to establish sensitivity. Figure 2 to 7 shows obtained Melt and HRM profiles generated for known *Plasmodium* species which were subsequently used as reference profiles for each species and used for analysis of known patient samples from the two health centres.

Table 2 below shows average melting peak values obtained from six different runs done in the three samples with known *Plasmodium* species. The Tm values were highly reproducible across six different melt curve runs.

Table 2**: Melting temperature curve peak values for three *Plasmodium* species**

Runs	**Average T_m peak values ± SD in ^oC**		
	Plasmodium falciparum	*Plasmodium malariae*	*Plasmodium ovale*
1	78.78	77.61	80.69
2	79.07	77.65	81.11
3	79.22	77.62	80.94
4	78.72	77.70	81.12
5	78.84	77.40	80.63
6	78.87	77.45	81.09
Mean average	**78.91±0.19**	**77.57±0.12**	**80.93±0.22**

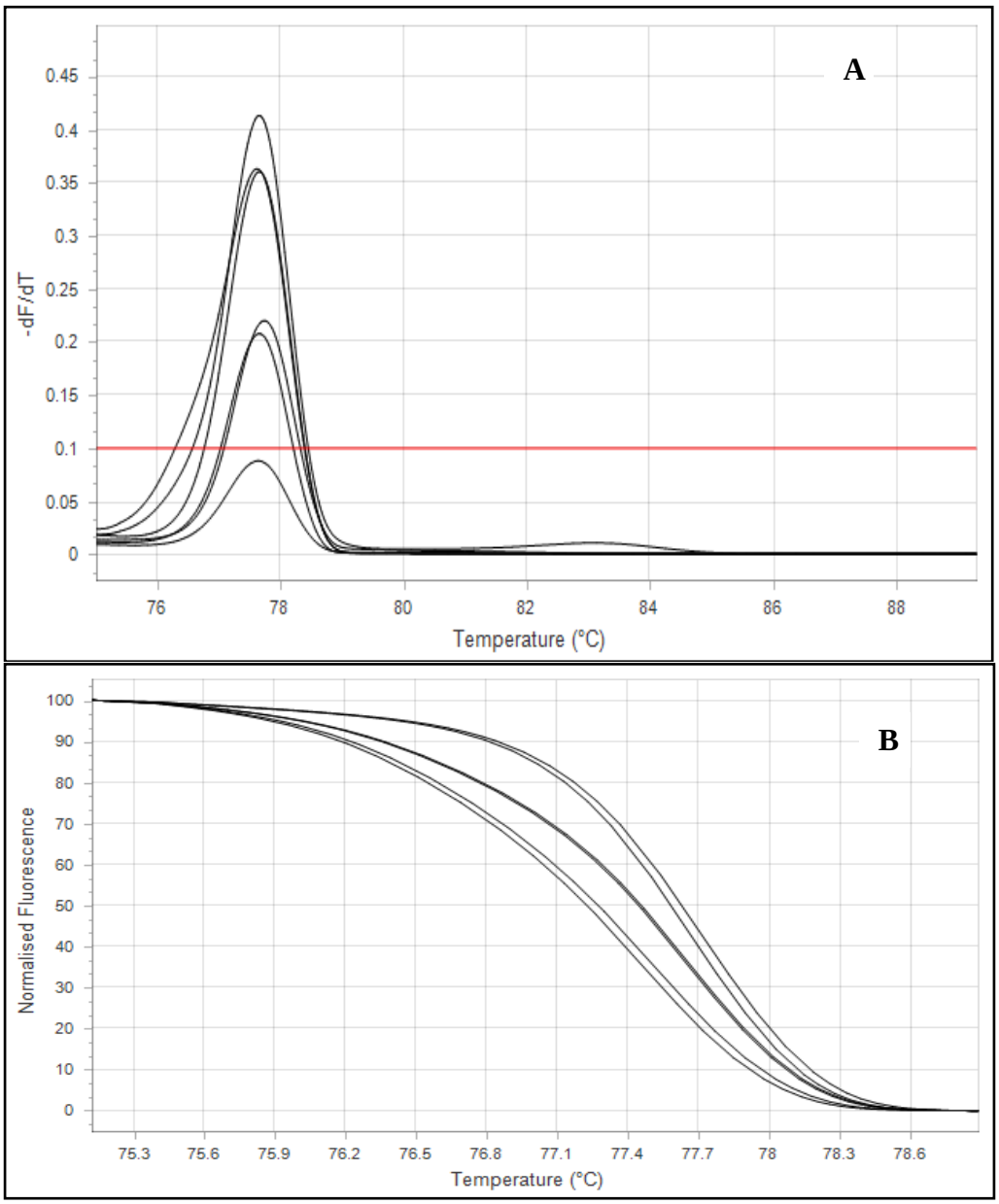

Figure 2: Representative amplification plots (A) Melt curve (B) Normalised HRM curves analysis for PCR-HRM *Plasmodium malariae* reference profiles

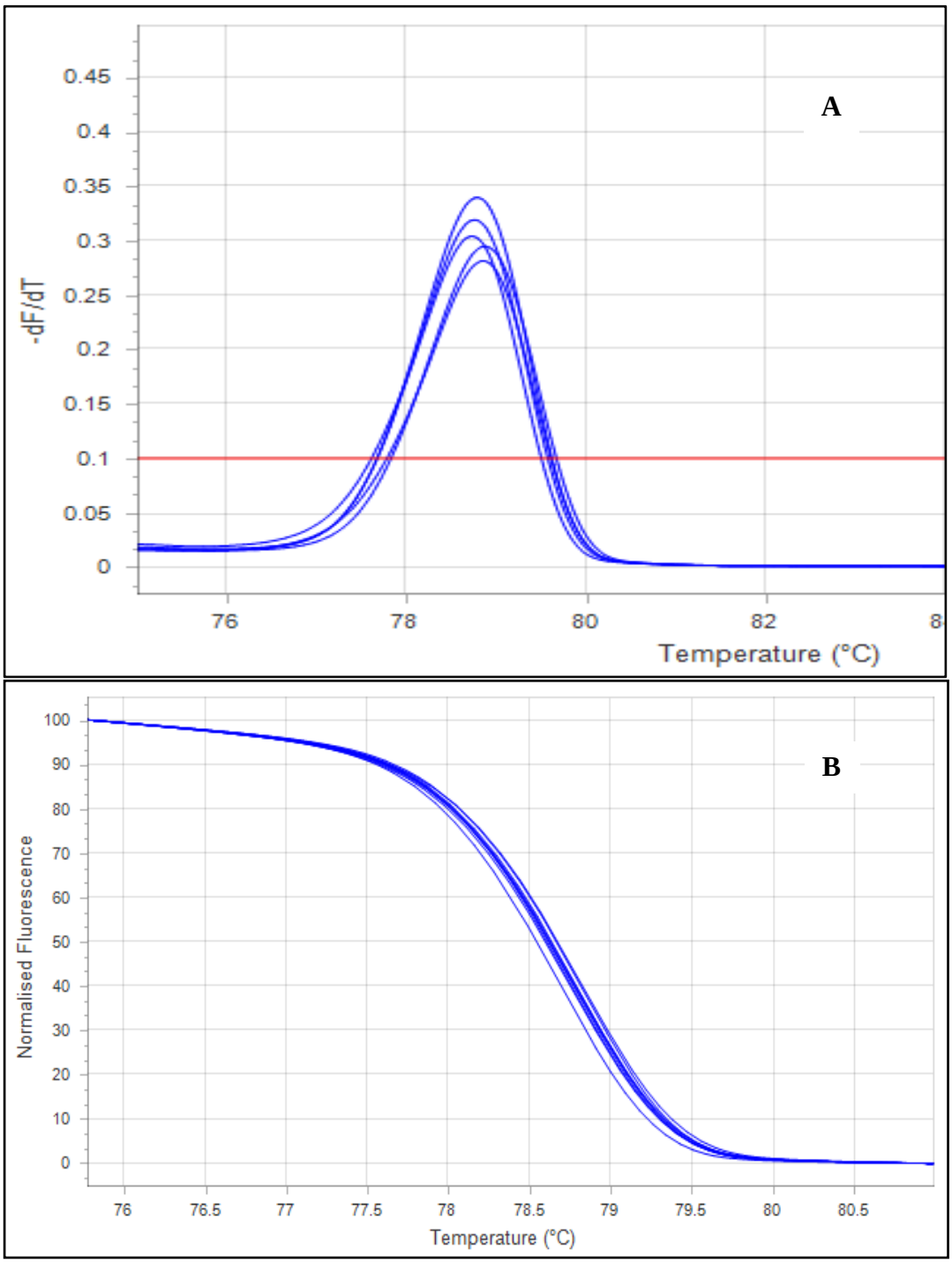

Figure 3: Representative amplification plots (A) Melt curve (B) Normalised HRM curves analysis for PCR-HRM *Plasmodium falciparum* reference profiles

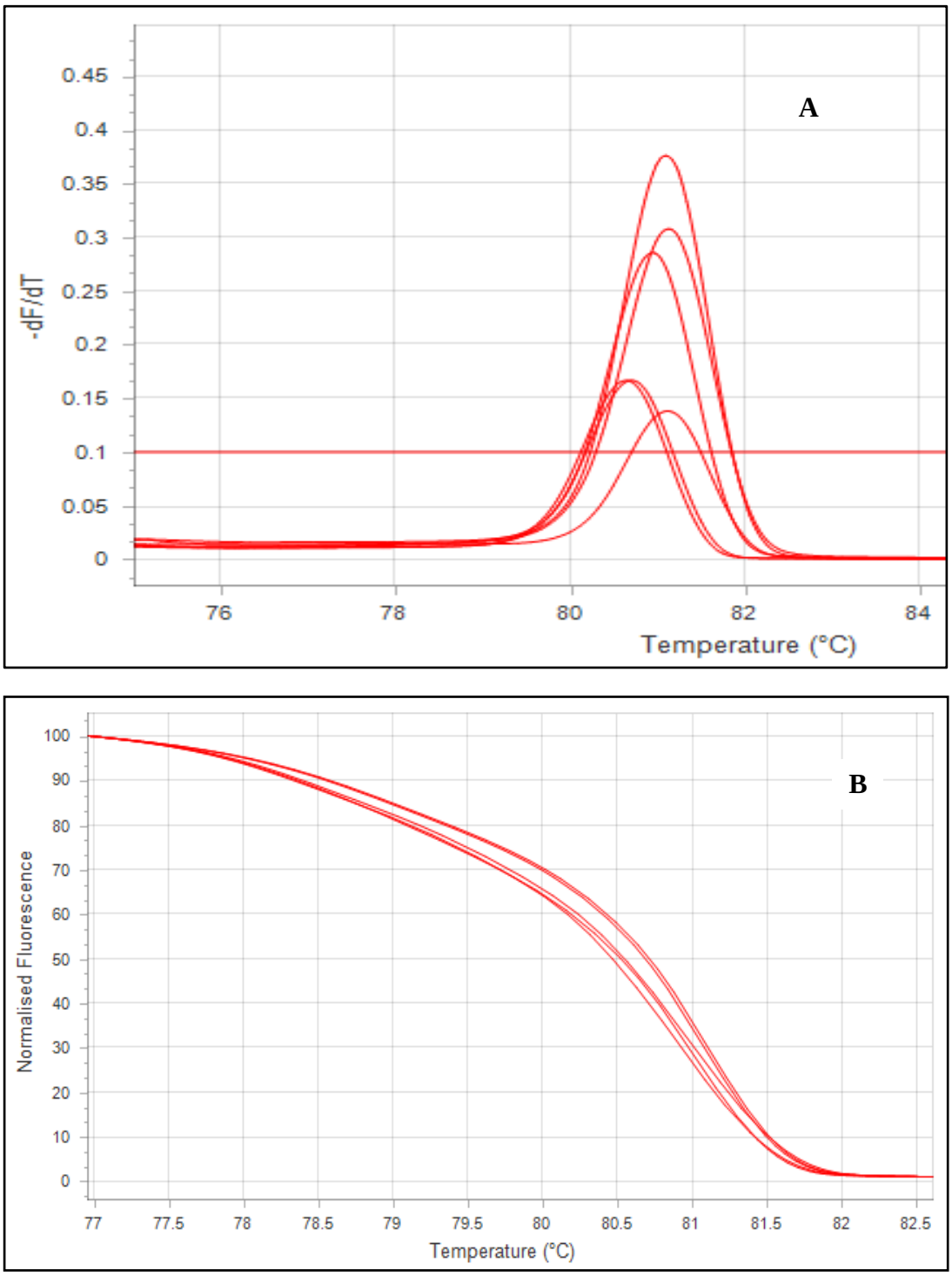

Figure 4: Representative amplification plots (A) Melt curve (B) Normalised HRM curves analysis for PCR-HRM *Plasmodium ovale* reference profiles

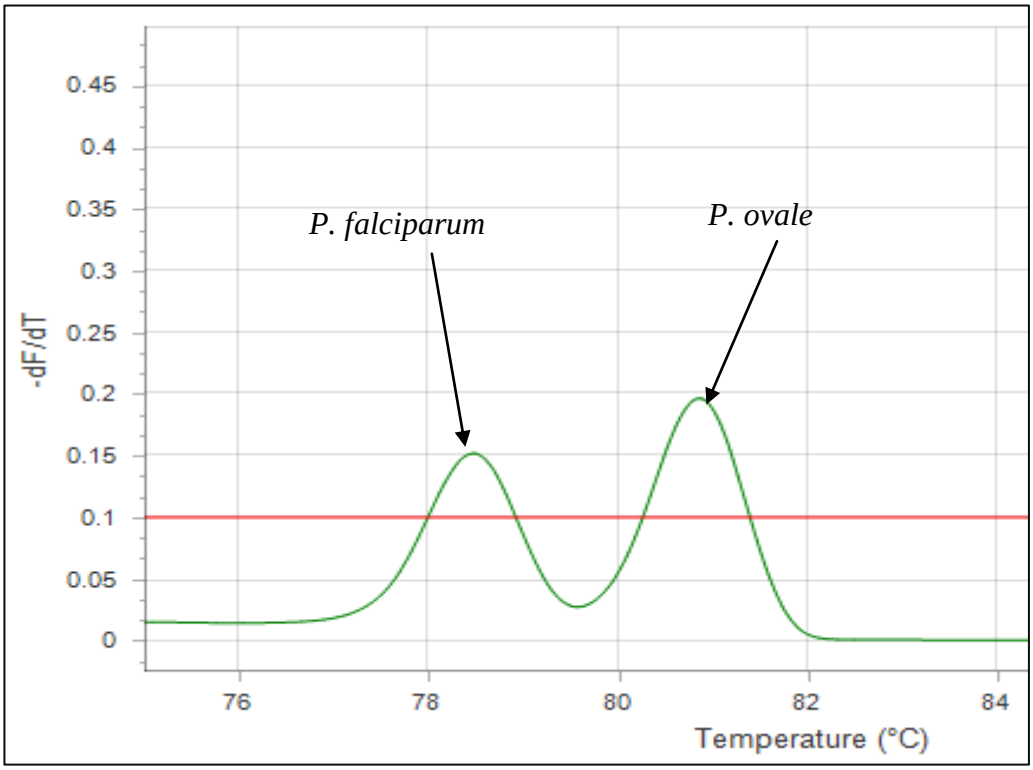

Figure 5: Melt curve analysis for PCR-HRM mixed infection (*Plasmodium ovale* and *Plasmodium falciparum*) reference profiles

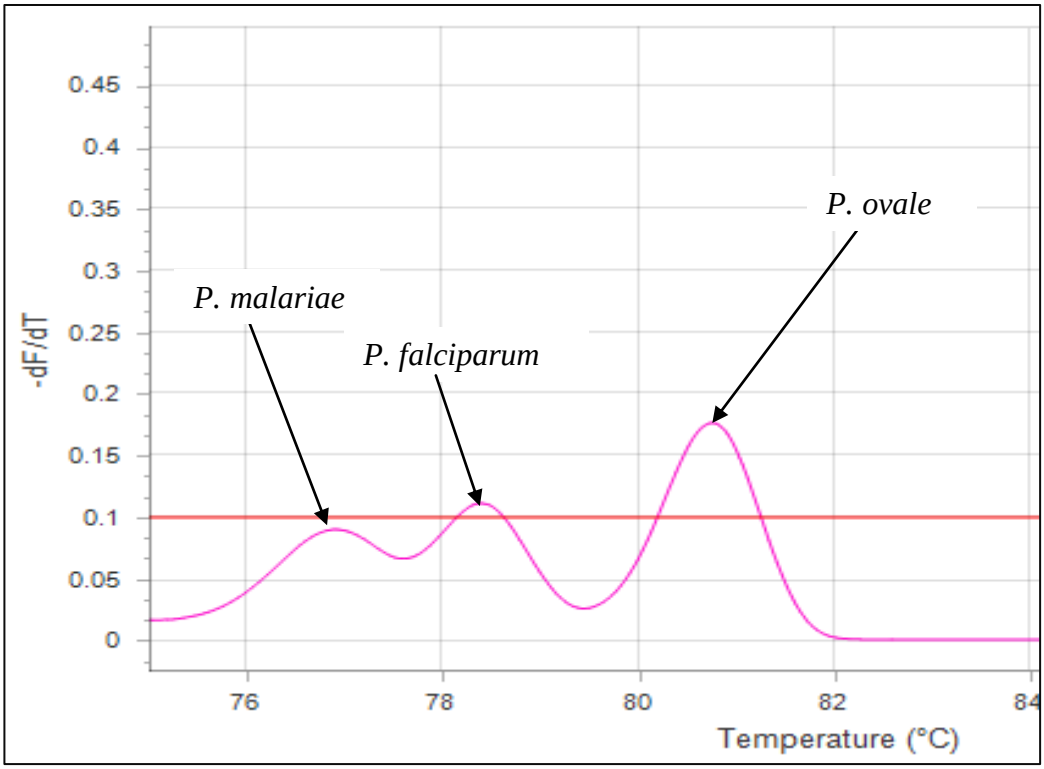

Figure 6: Melt curve analysis: PCR-HRM reference profiles for mixed infection (*Plasmodium malariae, Plasmodium ovale* and *Plasmodium falciparum*)

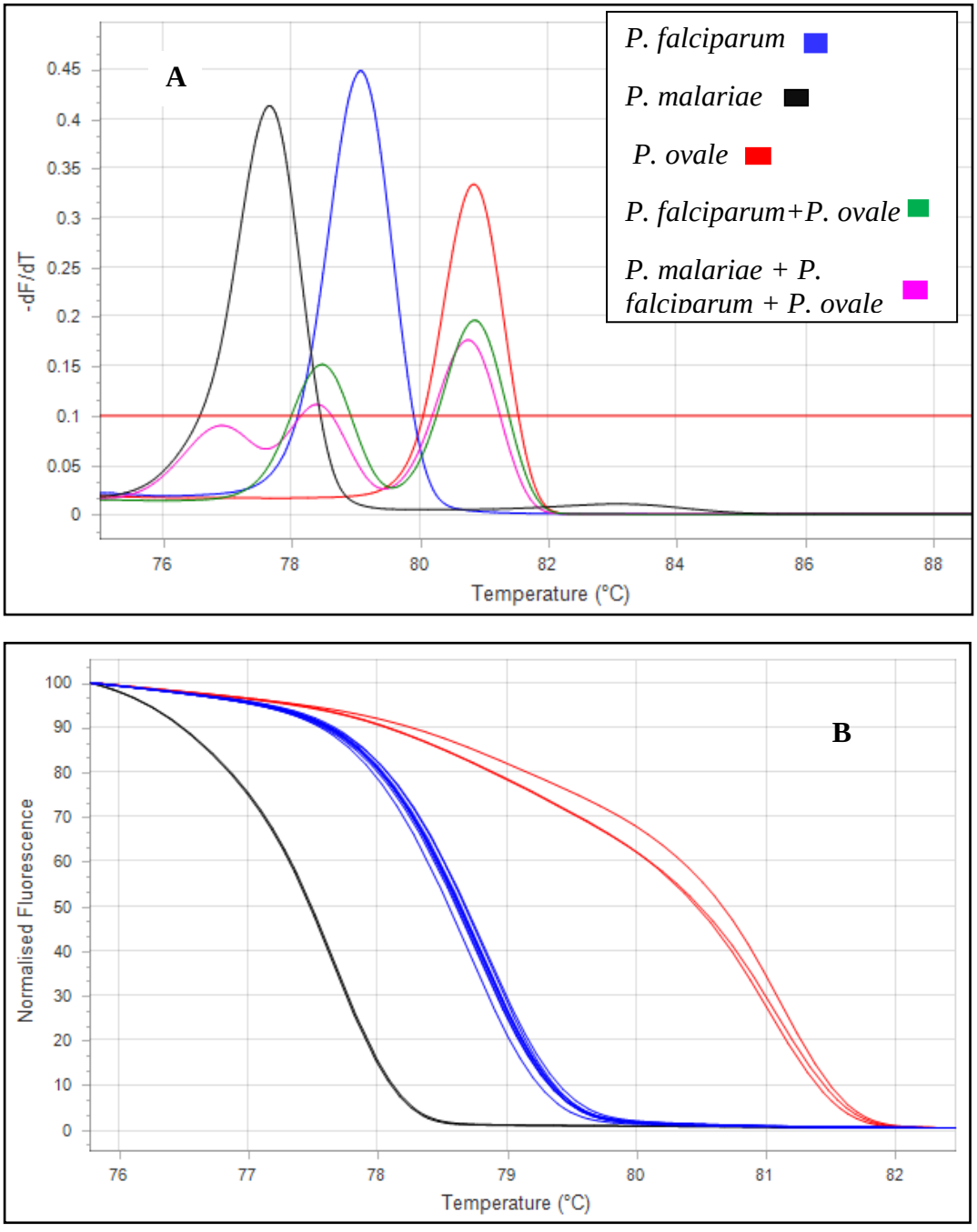

Figure 7: Representative amplification plots (A) Melt curve (B) Normalised HRM curves analysis for PCR-HRM *plasmodium* species reference profiles

4.2 Comparative malaria diagnosis by using microscopy, mRDT and PCR-HRM techniques

4.2.1 Comparison of microscopy and PCR-HRM in malaria diagnosis

The overall prevalence of infections detected by microscopy and PCR-HRM were 11.43% (95% CI 6.68-17.90%) and 12.86% (95% CI 7.8-19.56%), respectively (Fig. 8). Among the 16 samples which had tested positive by microscopy at SUA Health Centre, only 11 (68.75%) tested positive with PCR-HRM while the rest (5 samples) 31.25% were negative. Also among the 124 samples which tested negative by microscopy, 8 samples (6.45%) were found to be positive when analysed by PCR-HRM.

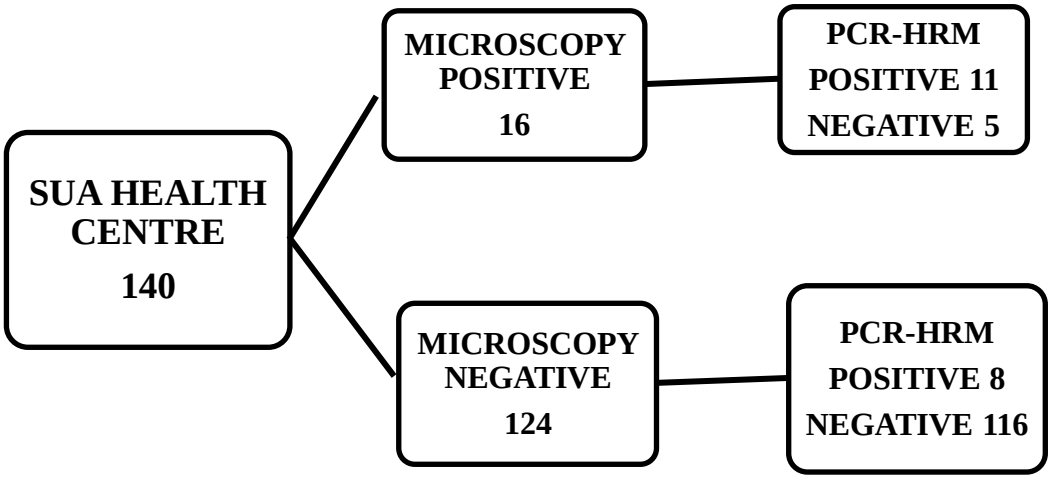

Figure 8: Comparative diagnosis of malaria infection generated by microscopy and PCR-HRM

The combined estimates of microscopy: PCR-HRM prevalence ratio was 0.9300 (95% confidence interval [CI], 0.4406–1.9628) with a $p \leq 0.0361$ suggesting a significant difference between the two diagnostic tests. The microscopy estimated sensitivity and specificity values against PCR-HRM are presented in Table 3. The

sensitivity of microscopy was less than the combined estimate of the microscopy: PCR-HRM prevalence ratio, mainly because of the very low specificity of microscopy (this is high numbers of false-positive results).

Table 3: Comparative sensitivity and specificity of Microscopy against PCR-HRM techniques in malaria diagnosis

	PCR-HRM +	PCR-HRM –	Total
Microscopy +	11	5	16
Microscopy –	8	116	124
Total	19	121	140
Sensitivity	0.5789 (95% CI 0.335-0.7975)		
Specificity	0.9587 (95% CI 0.9062-0.9864)		

4.2.2 Comparison of mRDT and PCR-HRM prevalence

The overall prevalence of infection detected by mRDT was 1.73% (95% CI 1.38-7.93%) and 4.97% (95% CI 2.17-9.56%) was detected by PCR-HRM (Fig. 9). Among the 6 samples which tested positive by mRDT at Sabasaba Health Centre, 5 were found to be positive when tested by PCR-HRM. Also among 155 samples which tested negative by mRDT, 3 samples tested positive by PCR-HRM.

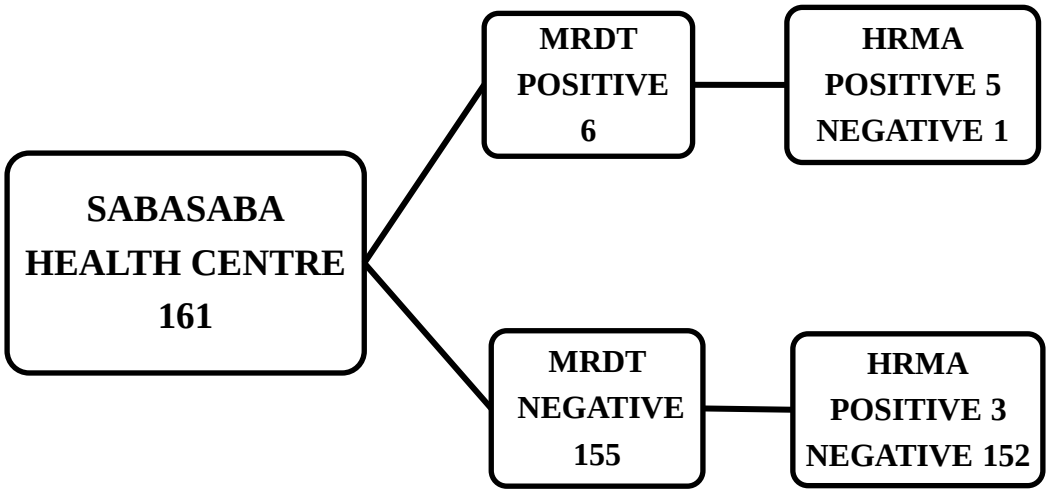

Figure 9: Prevalence estimates of malaria parasites generated by mRDT relative to those generated by PCR-HRM

The combined estimates of mRDT: PCR-HRM prevalence ratio was 0.7403 (95% confidence interval [CI], 0.2510–2.1840) with a $P \leq 0.2987$. The mRDT sensitivity and specificity values against PCR-HRM are presented in Table 4. The mRDT sensitivity estimate closely corresponded to the combined estimate of the mRDT: PCR-HRM prevalence ratio, mainly because of the very high specificity of mRDT (this is low numbers of false-positive results).

4.3 Plasmodium species identification

4.3.1 Species identified from SUA health centre

All 16 samples as diagnosed positive by microscopy at SUA health centre were all identified as *P. falciparum*. When the samples were analysed by PCR-HRM, only 11 samples were found to be positive with species identified being; *P. falciparum* 7 samples (Fig. 10), *P. ovale* 1 sample (Fig. 11), *P. malariae* 1 sample (Fig. 12), 2 samples were identified as mixed infection; one (1) involving *P. falciparum, P.*

malariae and *P.* ovale (Fig. 13) while the other involved *P. falciparum* and *P. ovale (*Fig. 14*)*. The 8 samples that were diagnosed negative by microscopy were found to be positive by PCR-HRM and identified as; *P. falciparum* 4 samples (Fig. 10), *P. ovale* 4 samples (Fig. 11).

Table 4: Comparative sensitivity and specificity of mRDT against PCR-HRM techniques in malaria diagnosis

	PCR-HRM +	PCR-HRM –	Total
mRDT +	5	1	6
mRDT –	3	152	155
Total	8	153	161
Sensitivity	0.7143 (95% CI 0.2904-0.9633)		
Specificity	0.9935 (95% CI 0.9644-0.9988)		

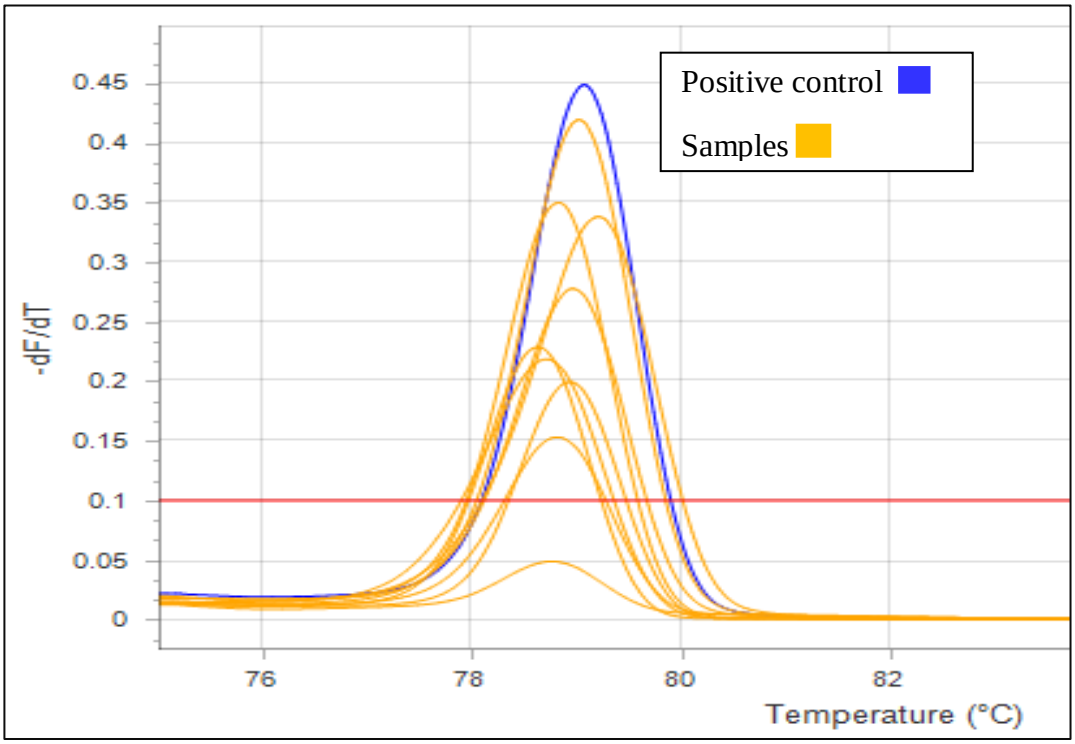

Figure 10: Melt curve analysis for patient samples from SUA hospital infected with *Plasmodium falciparum* as identified by PCR-HRM

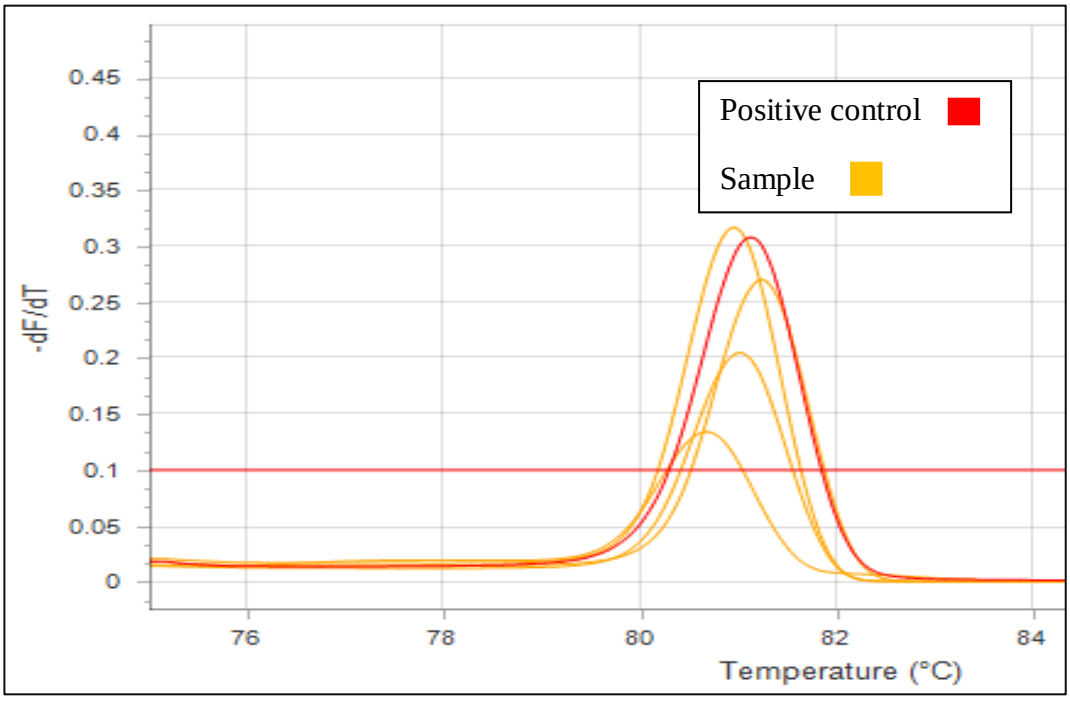

Figure 11: Melt curve analysis for patient samples from SUA hospital infected with *Plasmodium ovale* as identified by PCR-HRM

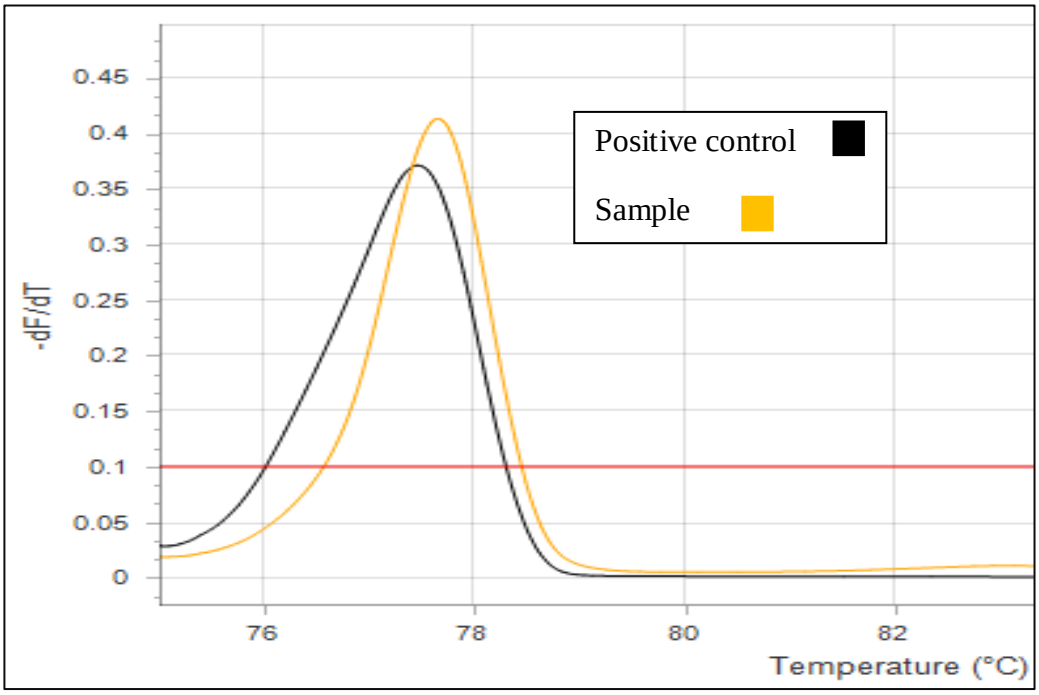

Figure 12: Melt curve analysis for patient samples from SUA hospital infected with *Plasmodium malariae* as identified by PCR-HRM

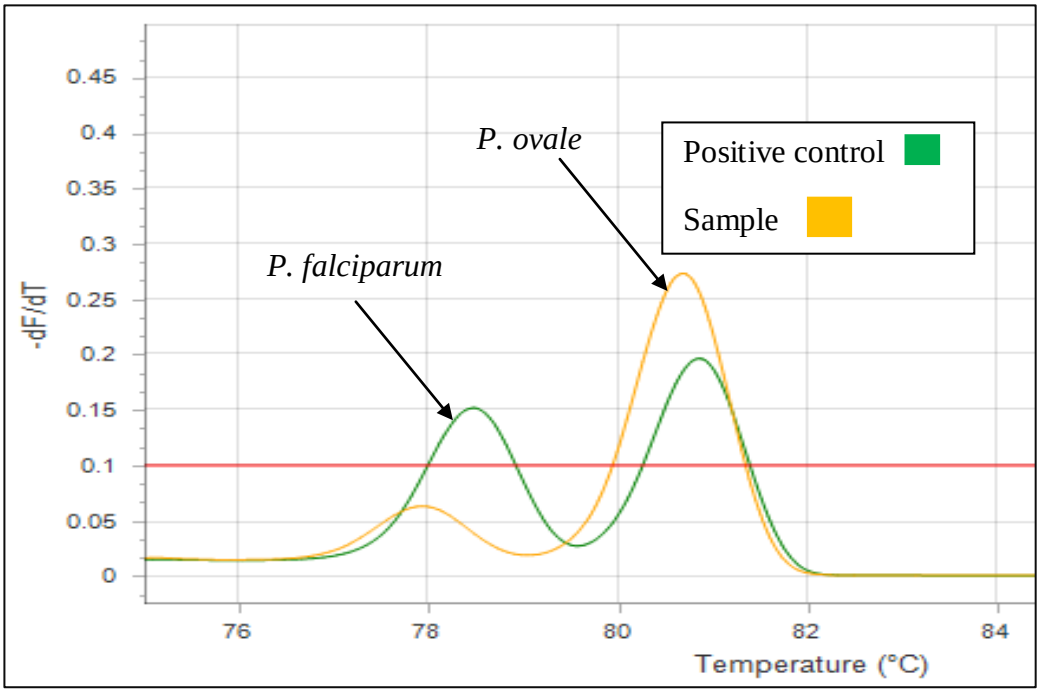

Figure 13: Melt curve analysis for patient's samples from SUA hospital involving mixed infection (*Plasmodium falciparum* and *Plasmodium ovale)* as identified by PCR-HRM

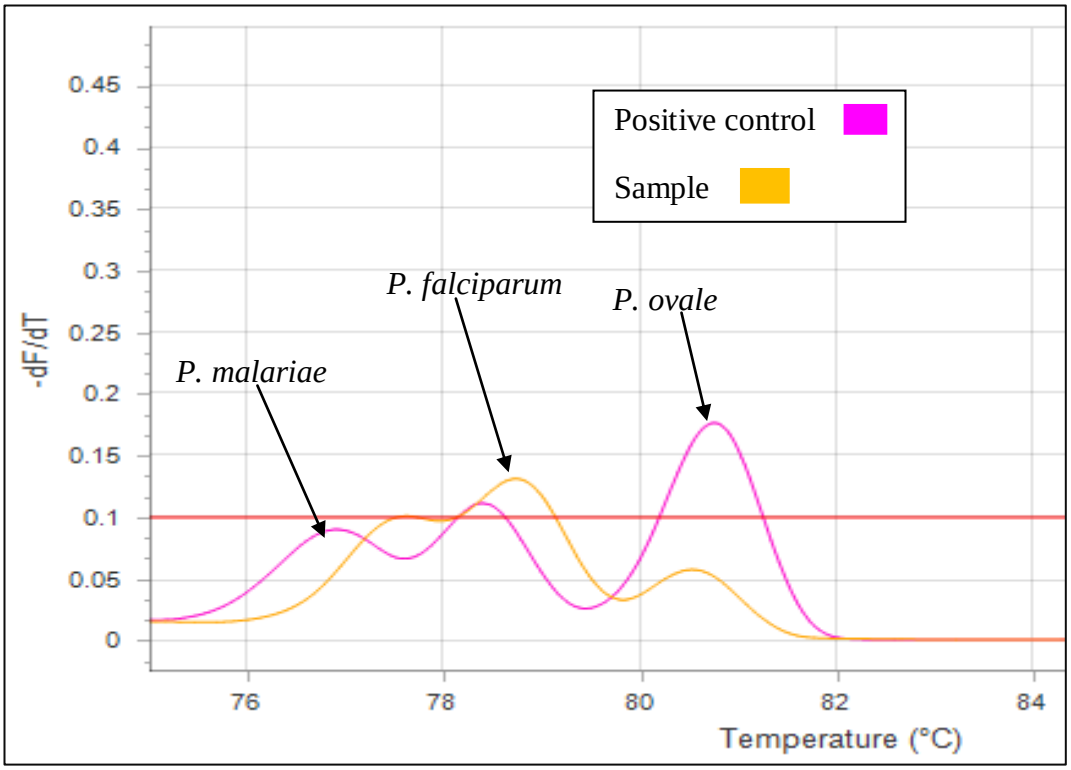

Figure 14: Melt curve analysis for patient's samples from SUA hospital involving mixed infection (*Plasmodium falciparum, Plasmodium malariae* and *Plasmodium ovale)* as identified by PCR-HRM

4.3.2 Species identified from Sabasaba health centre

Six (6) samples were diagnosed positive by mRDT at Sabasaba health centre whereby 5 samples were identified as *P. falciparum*, 1 sample identified as Pan (other species detected). When the same samples were diagnosed by PCR-HRM 5 samples were found to be positive with species identified as follows *P. falciparum* 4 samples (Fig. 15), 1 as mixed infection involving *P. falciparum* and *P. ovale* (Fig. 18). Three (3) species diagnosed negative by mRDT were found positive by PCR-HRM and identified as; *P. malariae* 2 samples (Fig. 16), and *P. ovale* 1 sample (Fig. 17).

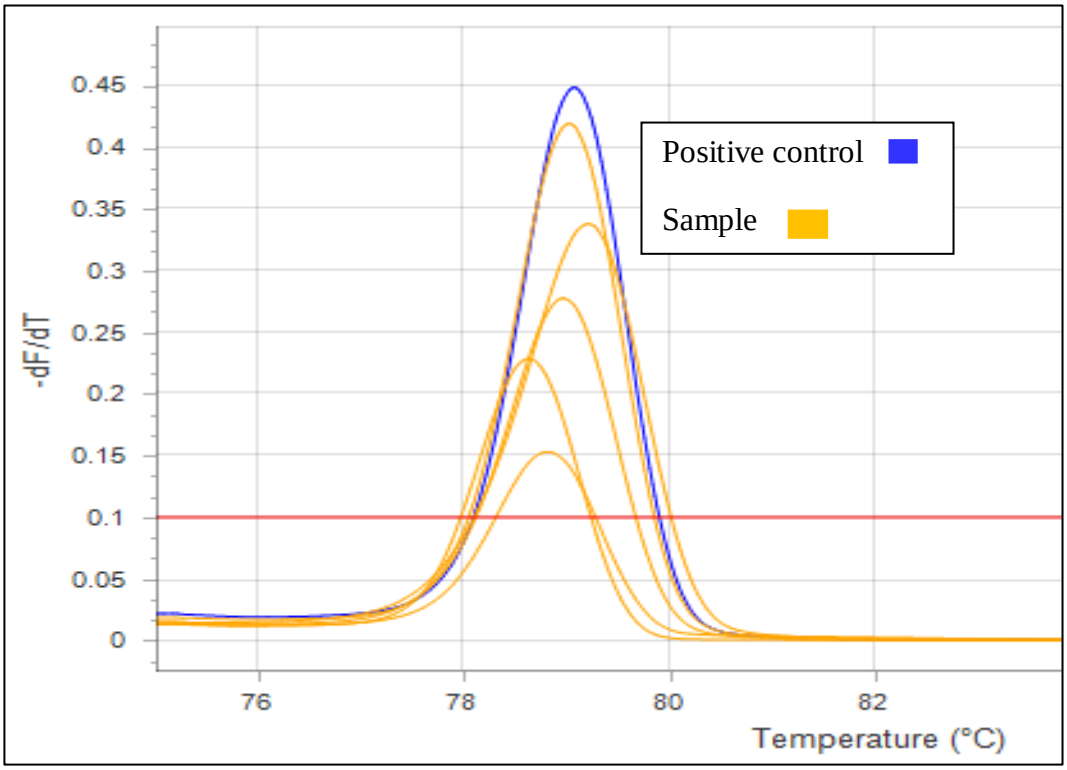

Figure 15: Melt curve analysis for patient samples from Sabasaba health centre infected with *Plasmodium falciparum* as identified by PCR-HRM

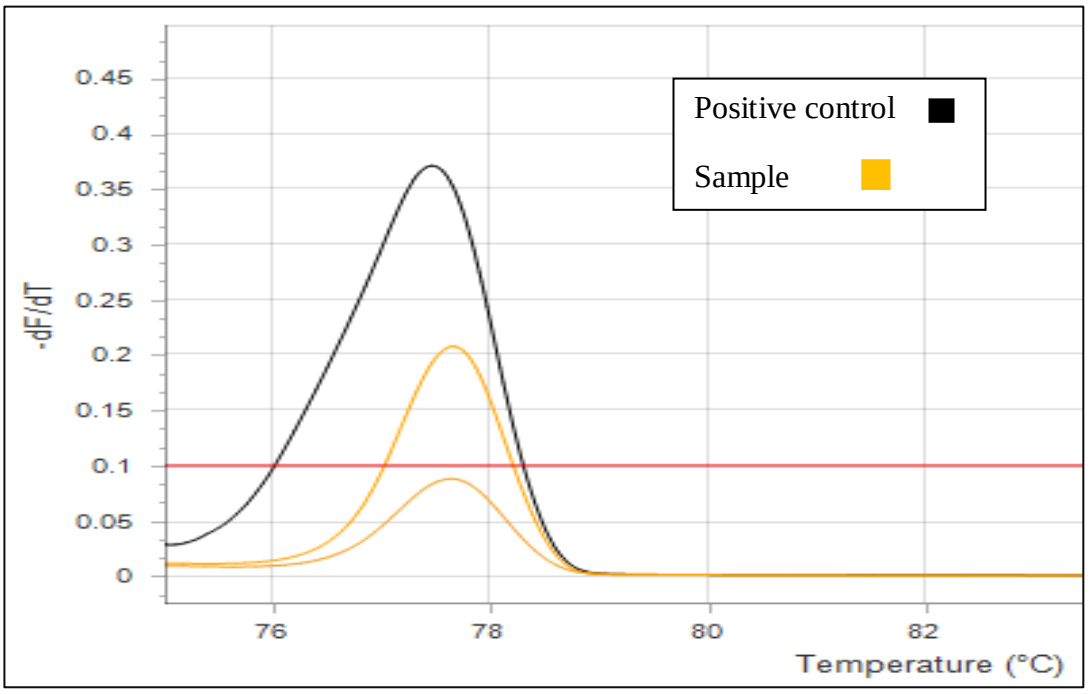

Figure 16: Melt curve analysis for patient samples from Sabasaba health centre infected with *Plasmodium malariae* as identified by PCR-HRM

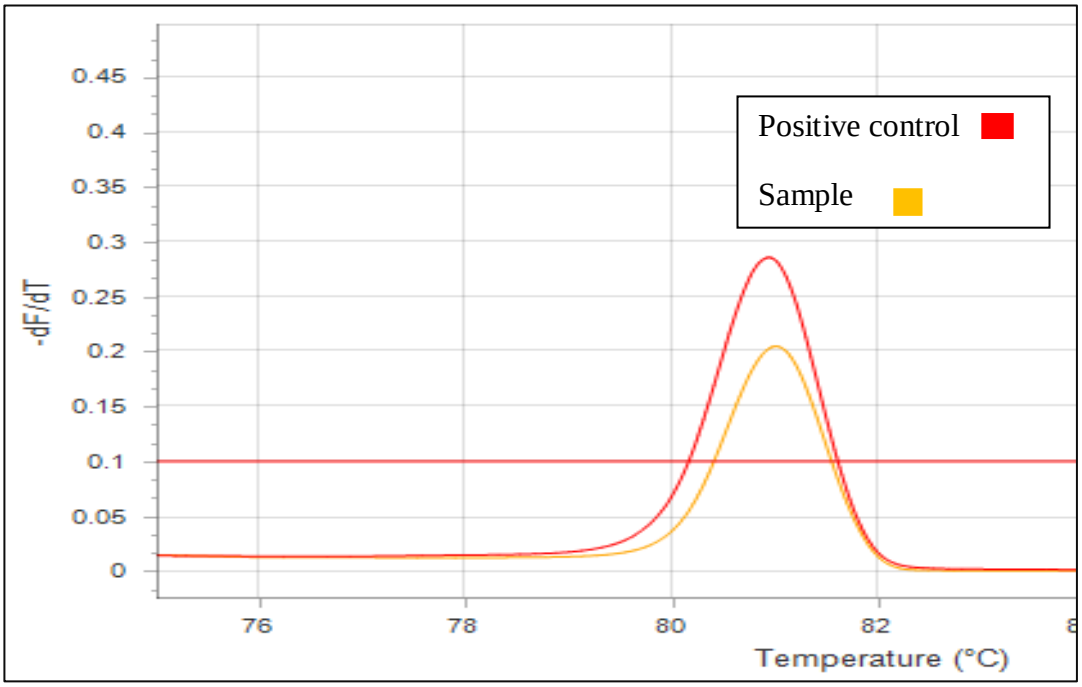

Figure 17: Melt curve analysis for patient samples from Sabasaba health centre infected with *Plasmodium ovale* as identified by PCR-HRM

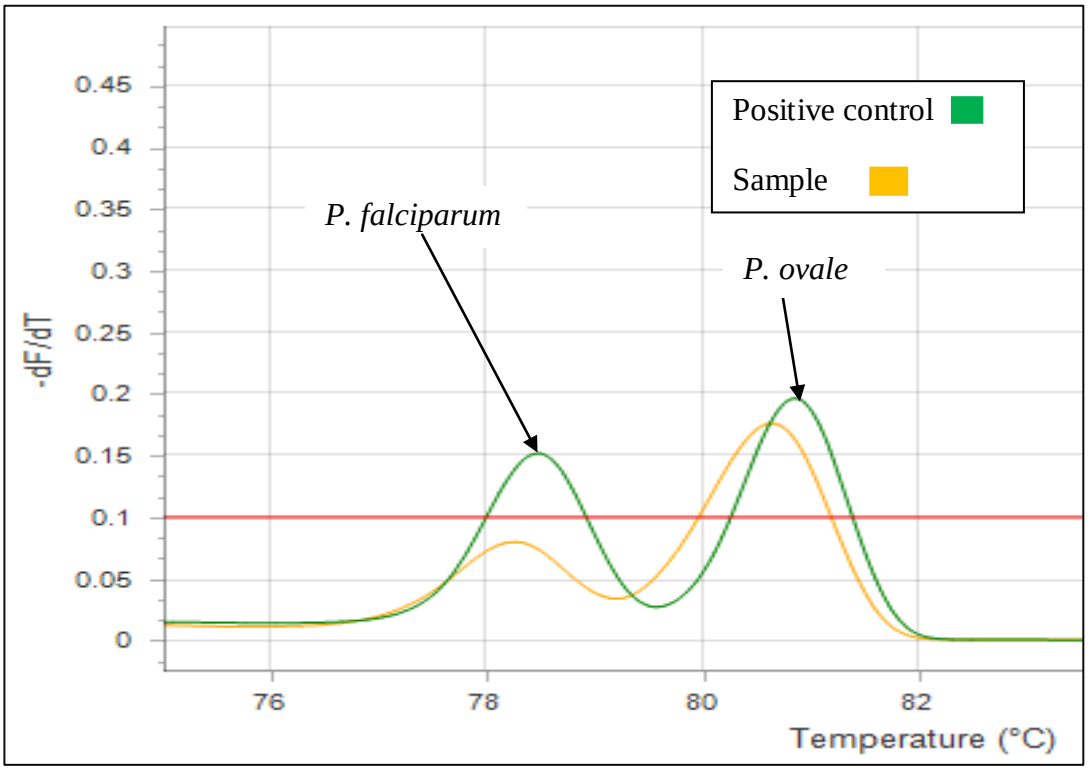

Figure 18: Melt profiles for samples from patients at Sabasaba health centre involving mixed infection (*Plasmodium falciparum* and *Plasmodium ovale)* as identified by PCR-HRM

CHAPTER FIVE

5.0 DISCUSSION

5.1 Overview

Humans can harbour single or mixed malaria infection with very low level of parasites, a phenomenon that is mainly attributed to partial immunity (Lau *et al*., 2015). Available methods used for malaria diagnosis in many health centres of malaria-endemic countries are microscope and antigen-based kits. However, they are deficient in detecting mixed malaria infections and low parasitaemia levels this in turn increases the risk of untreated malaria (Berzosa *et al.,* 2018; Mfuh *et al.,* 2019).

Studies reported by Wongsrichanalai *et al.* (2007) and Mangold *et al.* (2005) also confirmed the difficultly of using microscopy in the diagnosis of mixed malaria infections leading to misidentification of *Plasmodium* species when compared to other reference method (real time PCR and RDTs). Furthermore, a relatively low sensitivity of Histidine Rich Protein-2 based RDT commonly used for malaria diagnosis in the hands of health care workers was observed in Zanzibar, in a study done to assess the usefulness of RDT versus PCR for *Plasmodium falciparum* diagnosis (Shakely *et al.,* 2013).

This study was designed to compare the efficiency of PCR-HRM in the diagnosis of human *Plasmodium* species infections in relation to mRDT and microscopy. Dried blood spot (DBS) samples were collected routinely on a daily basis from two main health centres within Morogoro urban municipal; SUA health centre located in the main campus of Sokoine University of Agriculture (SUA) and Sabasaba health centre located centrally in Morogoro town. Sabasaba health centre heavily rely on

mRDTs for malaria diagnosis while SUA health centre depends on microscopy for diagnosis. All patients visiting the two health centres presenting with febrile illness were recruited in the study.

5.2 Standardizing and validating the PCR-HRMA technique

Dried blood spot (DBS) filter paper samples with known *Plasmodium* species *(P. falciparum, P. ovale and P. malariae)* obtained from NIMR laboratory at Tanga were extracted and used to standardize the PCR-HRM technique. Extraction was done using the Chelex Protocol adopted from Malisa *et al.* (2010) with some modifications. DNA concentration and quality for each sample was measured using Nano Drop spectrophotometer prior to nested PCR procedures. Since these analyses were being done for the first time in Tanzania on MIC, standardization of the methods previously reported by Kipanga *et al*. (2014) and intended to be adopted in this study were necessary prior to being used for analysis of patient samples. This included the optimization of annealing temperature, DNA template concentration of the primary and secondary PCR amplicons. The annealing temperature finally optimized and used in the PCR-HRM in the present study ranged between 66℃ to 54℃. DNA extract samples were diluted to give a DNA concentration ranging from 5 to 10ng/µl, a concentration range which provided best amplification results in the primary amplification step. The first amplification product was also diluted ten folds (10x) and used as DNA template for the secondary amplification.

Following PCR-HRM standardization, Melt and HRM profiles were established and validated for all three sets of *plasmodium* species *(P. falciparum, P. ovale and P. malariae)* obtained from NIMR. For each species, six different runs were done so as

to assess their reproducibility (see Table 2). Extracted samples were also combined to produce references for mixed species. The obtained Melt and HRM profiles generated for known *Plasmodium* species were subsequently used as reference profiles for each species (see Fig 2 to 7) and used for analysis of known patient samples. For each *Plasmodium* species sample distinct melt curve peaks were observed in this study and were comparable to those previously reported by Kipanga *et al.* (2014). Two peaks were clearly seen on the Melt curve plot when two species samples were combined. Similarly three peaks were observed when three species DNA were combined. These validations confirmed the ability of the nPCR-HRM to detect double and triple infection.

5.3 Comparative assessment of three malarial diagnostic methods (microscopy, mRDT and nPCR-HRMA)

In this study, patient samples which were analysed by nPCR-HRM were those previously obtained from SUA Health Centre to which patients were diagnosed to be malaria positive or negative by microscopy and those obtained from Sabasaba Health Centre to which patients were diagnosed to be malaria positive or negative by mRDT. The nPCR-HRM confirmed that 6.4% of the patients attending the SUA Health Centre and confirmed to be malaria negative were indeed positive. Similarly, 31.3% of patients' diagnosed positive to malaria by microscopy at the same Centre were negative by nPCR-HRM. Greater sensitivity and specificity of nested PCR over microscopy has been reported in many studies (Alemu *et al.,* 2014; Wang *et al.,* 2014; Lau *et al.,* 2015). The relative high level of malaria misdiagnosis by microscopy observed at the SUA Health Centre might either be Centre specific since

the precision of blood-smear diagnostic approach is usually compromised by the quality of the blood slide preparation, the number of microscope fields analyzed (blood volume), and the microscopists' expertise and experience (Doni *et al.*, 2016). These results however, are not uncommon in many urban and rural health clinics in malaria endemic regions which still depend on microscopy as a sore malaria diagnostic tool as it is not sensitive enough to detect low *Plasmodium* parasitaemia mostly observed in such patients (Kipanga *et al.*, 2014).

Compared to nPCR-HRM, overall Giemsa stained microscopy for malaria diagnosis had sensitivity of 0.5789 and specificity of 0.9587 with a $p \leq 0.0361$ suggesting a significant difference between the two diagnostic tests. Lower sensitivity of microscopy in diagnosing other species was probably due to presence of artifacts, low quality of stained smears, low parasitaemia in mixed infection and high prevalence rate of *P. falciparum* malaria in the study area.

Among patients visiting Sabasaba Health Centre for malaria diagnosis, results showed that 1.29% of patients who were diagnosed to be malaria negative by mRDT at the centre were indeed malaria positive by nPCR-HRM while 16.7% of patients who were malaria positive by mRDT were found to be negative by nPCR-HRM. About 83.3% of mRDTs results from the Sabasaba Health Centre were in agreement with the nPCR-HRM results obtained in this study (0.7143 sensitivity, 0.9935 specificity); indicating that mRDTs were capable of providing more accurate diagnosis of *Plasmodium* parasites compared to microscopy. In the presence of well trained microscopists, microscopy has however proven to be a better malaria diagnosing tool than RDT (Oyetunde *et al.*, 2015). In another study done in Nigeria

by Azikiwe *et al.* (2012), to compare the efficiency of microscopic method and rapid diagnostic kits in analysing 200 patients presenting themselves with malaria signs, 128 out of 200 tested positive to RDTs based on malaria antigen (whole blood) method (64%) similar to the kit type used in this study, while 118 out of 200 patients tested positive to visual microscopy of Lieshman and diluted Giemsa (59%). Both studies above confirm the usefulness of using RDT based on malaria antigen over microscopy in malaria diagnosis.

However, RDTs are known to produce false positive results because of residual antigen, which can persist for weeks after treatment and parasite clearance Mixson-Hayden *et al.* (2010). RDTs also fail to determine the magnitude of parasitaemia. A study done by Shakely *et al.* (2013) indicated a relatively lower sensitivity of RDT which was partly contributed by the health care workers performance and a prozone effect *i.e.* false-low results in immunological tests. Due to an excess of either antigens or antibodies, RDTs are also reported to fail in detecting some infections with lower numbers of malaria parasites circulating in the patient's bloodstream (CDC, 2018). As much as RDT has proven to be a quick and good diagnostic malaria technique, Berhane *et al.* (2017) confirmed high false-negativity rate in PfHRP-2-detecting RDTs and this was not associated with the quality of RDTs, storage, handling and operating errors.

5.4 *Plasmodium* species identification

Plasmodium falciparum was identified to be the only infecting species in all 16 patients who tested positive by microscopy at SUA Health Centre. PCR-HRM confirmed 11 patients to be malaria positive and parasites identified as; *P. falciparum*

(7 patients), *P. ovale* (1 patient), *P. malariae (*1 patient) all as single infections. Two (2) patients had mixed infections *P. falciparum, P. malariae* and *P. ovale* (1 patient) and *P. falciparum* and *P. ovale* in another patient. Among the 124 patients proven malaria negative microscopically at SUA Health Centre, 8 (6.4%) were found to be PCR-HRM positive with infecting *Plasmodium* species identified as; *P. falciparum* (4 patients) and *P. ovale* (4 patients). Despite the fact that blood film microscopy remains to be the standard diagnostic method for malaria, previous studies have shown that even with experienced microscopists', missing detection occurs particularly in cases of mixed infection or low parasitaemia Hanscheid (2003) and Payne (1988) a case observed also in this study.

At Sabasaba Health Centre, *P. falciparum* infection was confirmed in 5 out of 6 patients who tested positive by mRDT, and one patient was diagnosed to be infected with other *Plasmodium* species in a pan specific manner. All four (4) patients who tested malaria positive by mRDT at Sabasaba Health Centre and later analysed with nPCR-HRM were identified to harbour *P. falciparum* by both diagnostic methods. One (1) patient who had other type of *Plasmodium* species detected by mRDT in a pan specific manner was confirmed by nPCR-HRM to be infected with *P. falciparum* and *P. ovale* as a mixed infection. Likewise the three (3) patients who were RDT negative at Sabasaba health centre were found to be nPCR-HRM positive infected with *P. malariae* (2 patients) and *P. ovale* (1 patient). Studies show that occasionally, when *P. falciparum* parasitaemias are elevated, RDTs give false positives of non-falciparum species (Berzosa *et al.,* 2018). This calls for improved systems for RDT

supervision and quality control in primary health care facilities, but also for more sensitive point-of-care malaria diagnostic tools (Shakely *et al.,* 2013).

5.5 Mixed-species infection detection

Molecular diagnostic tools such as real time PCR (Mangold *et al.,* 2005*),* nested PCR (Shakely *et al.,* 2013) have provided highly sensitive detection tools for malaria diagnosis of species involved in mixed infections. Likewise, high level of sensitivity has also been observed by combining nested PCR with real-time species differentiating HRMA in accurate reporting of *Plasmodium* infection prevalence that includes mixed and low-parasitaemia infections (Kipanga *et al*., 2014). The use of nPCR-HRMA in this study has shown a very high level of sensitivity in detecting single and mixed infections in the study population who were misdiagnosed by microscopy and mRDT at the two study Health Centers.

The current focus in malaria diagnosis by microscopy and mRDTs and treatment in Tanzania is manly directed towards *P. falciparum*. There is a substantial lack of data on local transmission patterns of the neglected malaria parasites *P. malariae* and *P. ovale* species. In a recent report which analyzed 2897 patient blood samples between 1994 and 2016 using a species-specific real-time PCR in evaluating temporal trends in the prevalence of all human infections with *P. falciparum, P. malariae, and P. ovale* species in Nyamisati has demonstrated the persistent transmission of *P. ovale* and to a lesser extent *P. malariae* despite a continued decline in *P. falciparum* transmission in the area (Yman *et al.*, 2019). Nyamisati is a rural fishing village located 150 km south of Dar es Salaam in the Rufiji river delta area in Kibiti District.

The current report by Tanzania National Bureau of Statistics 2017 showing a decline trend in malaria incidences is probably linked to the decline in *P. falciparum* infection, since the current diagnostic methods are not specie-sensitive enough to identify other non-*falciparum* species. Since the transmission patterns of the non-*falciparum* species has been observed to not follow those of *P. falciparum* Yman *et al.* (2019), there is a need for putting more attention towards non-falciparum malaria transmission patterns in Tanzania. This is more apparent to the prevalence of *P. ovale* and *P. malariae* which were observed in this study and never described before in the study area. Improved malaria diagnostic methods like the use of more cheaply and fast nPCR-HRMA, used in this study might help in the better understanding of the epidemiology of *P. malariae* and *P. ovale* species and, with a particular focus towards identifying asymptomatic carriers of infection and designing appropriate diagnostic method.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

The objectives of this study were to diagnose human *plasmodium* species infections using a high throughput molecular technique which combines the power of nested PCR and High Resolution Melting analysis. Specifically the study intended to (i) standardize and validate the PCR-HRMA tool for use in malaria diagnosis (ii) assess the ability of HRMA in the accurate identification of plasmodium species in single or mixed infections among febrile patients in the selected health centres of Morogoro region (iii) compare the relative sensitivity and accuracy of nPCR-HRM, microscopy and RDT in the screening of blood samples from febrile patients in the selected health centres. A total of 301 samples were collected from patients, visiting the two health centres (SUA and Sabasaba), indeed all patients were clinically suspected of malaria, presenting with febrile illness and laboratory analysis were conducted to obtain data of interest. Data were then analysed using EPI info version 7 and IBM SPSS version 20 software. This chapter presents conclusions and recommendations emanating from the major findings of the study.

6.1 Conclusions

In this study, 12% of the target population had mixed-species infections. Although these malaria infections are sub patent, they remain transmissible, complicating attempts to eradicate malaria in endemic regions. In addition, malaria-infected patients can present with unusual clinical features in cases of immunity or ant malarial drug resistance (Singh, 2004), but mRDTs and microscopy are capable of detecting parasitaemia of up to 50–100 p/mL (Bottius *et al.*, 1996). Nevertheless,

there remains a lack of awareness concerning these atypical manifestations, which are often diagnosed late or not at all, ultimately resulting in severe complications or death.

The accuracy of results observed in mRDT with reference to PCR-HRM was high; there was only one discordance results in species identification. The accuracy of results observed in microscopy with reference to PCR-HRM was relatively low, microscopy was only able to detect *P. falciparum* infection, and in most cases species identification was problematic. Molecular method (PCR-HRM) used in this study offered excellent specificity and sensitivity and could be considered as a reference standard for malaria infection diagnosis. It is a promising tool for simultaneous detection and discrimination of human infecting *plasmodium* species.

6.2 Recommendations

Due to the lower sensitivity of microscopy in identifying *Plasmodium* species, those with previous history of malaria and relapse cases should be diagnosed by RDT or PCR. The effectiveness of malaria microscopy depends on maintaining a high level of staff competence and accurate performance at all levels, inaccuracy of species diagnosis highlights the need of training and refresher courses from microscopists.

To reduce malaria morbidity and mortality, early diagnosis and prompt treatment are essential. As the choice of treatment usually relies on the exact diagnosis, misdiagnosis will lead to mismanagement. It has been suggested that nested PCR currently represents the most appropriate technique for detecting malaria in endemic areas containing a variety of *Plasmodium* species (Lau et *al.,* 2015).

Using molecular technique (PCR-HRM) enabled the identification of a large number of parasite infections. Thus it is important to review the status of tools used to characterize malaria infections, as well as the interpretation of data generated by these tools and some of the practical issues that will continue to confront malaria control efforts (Zimmerman, 2004). Comparative studies evaluating available diagnostic assays are required to determine the most efficacious methods so as to reduce malaria transmission and morbidity.

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APPENDICES

Appendix 1: Chelex Protocol for Plasmodium species DNA extraction from filter paper

1. Cut a sector of sample from the filter paper. Use sterile blade for this.
2. Place it in a 1.5ml eppendorf with 1ml of 0.5% saponin in 1x PBS.
3. Leave at 4ºC overnight. Haemoglobin is released into the wash leaving parasite DNA on paper.
4. Remove saponin and debris using pipette spin down to get all liquid (1min at 10,000g RCF)
5. Wash 2 times by adding 1ml of 1X PBS and spin for 1min at 10,000g RCF to remove liquid and debris.
6. Add 100µl of 20% chelex mix and incubate for 15min at 90ºC
7. Centrifuge for 5min to spin down chelex (10,000g RCF)
8. Take off DNA supernatant with pipette and discard the chelex and the paper.

Reagent preparation

a. Chelex preparation
   - Make up to 20% Chelex 100 (Sigma-Aldrich) in PCR quality water.
   - Adjust pH to 9.5 by adding 5M NaOH

b. PBS preparation.

   PBS should contain the following components in distilled water.
   - 137mM NaCl
   - 2.7mM KCl
   - 10mM Na_2HPO_4

- 1.8mM KH_2PO_4

Adjust pH to 7.4 (7.2 to 7.6)

Appendix 2: Age-malaria status characteristics of study participants

Age	Malaria status		Total
	negative	positive	
0-4	79	3	82
10-14	13	1	14
11-14	1	0	1
15-19	14	0	14
20-24	21	2	23
25-29	16	0	16
30-34	12	1	13
35-36	1	0	1
35-39	13	3	16
40-44	17	3	20
41-44	1	0	1
45-49	11	1	12
5-9	36	1	37
50-54	15	1	16
55-59	7	0	7
60-64	9	3	12
65-69	7	2	9
70-74	3	0	3
75-79	2	1	3
80-84	1	0	1
Total	279	22	301

Appendix 3: Informed Consent

Title: MOLECULAR DETECTION OF HUMAN MALARIA PARASITES USING HIGH RESOLUTION MELTING ANALYSIS IN MOROGORO REGION, TANZANIA

Introduction

Malaria being a global concern is an urgent public health priority. Tanzania has the third largest population at risk of malaria in Africa; over ninety percent of people live in areas where there is malaria. To facilitate proper management of malarial cases, it is prudently necessary to ensure that appropriate diagnosis is done. Molecular techniques are generally superior to antigen-detection tests and microscopy for detecting mixed infections and low parasitaemic infections in febrile patients. The current envisaged study aims at using (qPCR-HRMA) tool for the first time in Morogoro, Tanzania for malaria surveillance.

Study aim

Comparative molecular diagnosis of human plasmodium species infections using a high throughput High Resolution Melting Analysis (HRMA) tool versus the existing microscopic and malaria Rapid Diagnostic Tests (mRDTs) in Morogoro region.

Clarification and study procedure

If you agree to participate in this study, we will enrol you and ask you some few questions concerning this deadly disease. We will then take your temperature to check for a fever. If you have a fever above 37.5 ^{0}C, the health attendant will give you medicine to reduce the fever. Finger prick blood sample will be taken for malaria parasite diagnosis by mRDTs and microscopy. Filter paper blood spots will be taken for molecular biology assays. If you are found to have a more serious illness or need other medicine, you will receive treatment according to National guidelines.

Payment

You will not receive any direct payment for taking part in this study. It should not cost you any money to take part in this study.

Benefits

If the tests done in the study show that you have malaria, you will receive treatment. The information from this study will help us to better understand malaria disease in this community. This will provide important information on appropriate method for diagnosis, for future benefits of the community and country at large.

Access to my personal information

If you decide to take part in the study, the health attendant and I, will collect medical and personal information about you during the study. The hospital, members of the ethics committee or the review board for the study, auditors or regulatory authorities, will have access to this information in order to check that the study is done properly. All the information will be keep in confidential

Contact to answer any questions regarding this study

If you have any questions about this study or in case of illness related to taking part in the study, you should contact:

Professor Benezeth Mutayoba phone number 0762411406 or study PI Ms. Rehema Makoy phone number 0753653946. You can also address a letter to the Chair Person, Medical Research Coordinating Committee P.O.Box 9653. Dar es Salaam. Email: headquarters@nimr.or.tz

CONSENT STATEMENT

I declare that I have understood what the study is all about and I had been given enough information more over my question has been answered properly. I'm willing and consenting to participate in this study.

Participant's name: .....

Signature/Thumb-print: .....

Date: .....

Place/Village: .....

In case the participant is illiterate or a child

Guardians' name: ..... Signature: .....

Date: ..... Place/Village: .....

Researcher's name: ..... Signature: .....

Date: .....

Appendix 4: Fomu ya Ridhaa

Kichwa cha habari: UCHUNGUZI WA VIMELEA VYA MALARIA KWA KUTUMIA NJIA YA KISASA YA KIMOLEKULI WILAYANI MOROGORO, TANZANIA

Maelezo ya Utangulizi

Ugonjwa wa malaria ni janga kubwa la kimataifa na kipaumbele cha sekta ya afya. Katika bara la Afrika, Tanzania ni nchi ya tatu yenye idadi kubwa ya watu hatarini kupata malaria, zaidi ya asilimia tisini ya watu wanaishi katika maeneo ambayo kuna malaria. Ili kuwezesha usimamizi sahihi wa matukio ya malaria, ni muhimu kuhakikisha kuwa uchunguzi unaofaa unafanyika. Mbinu za molekuli ni bora zaidi kuliko vipimo vya kugundua antigeni na microscopy kwa kuchunguza maambukizi ya mchanganyiko na maambukizi ya chini ya vimelea katika wagonjwa wa kawaida. Utafiti uliopangwa sasa una lengo la kutumia mbinu ya kisasa zaidi (qPCR-HRMA) kwa mara ya kwanza mkoani Morogoro, Tanzania kwa ufuatiliaji wa malaria.

Madhumuni

Utafiti huu una lengo la kuchunguza vimelea vya malaria kwa kutumia mbinu ya kisasa ya kimolekuli (HRMA) na kulinganisha ubora wake na mbinu zinazotumika sasa (mRDTs na microscope).

Taratibu

Endapo utakubali kushiriki katika utafiti huu, utapata nafasi ya kuonana na kuongea na daktari ambaye atachunguza afya yako kwa kina na baada ya hapo utaombwa kufika kwenye sehemu ya wataalamu wa maabara kwa ajili ya kuchukuliwa vipimo. Utatolewa damu kwenye ncha ya kidole cha kati/chanda (Mkono wa kushoto) kwa kutumia kitoboleo maalum ili kupata kiasi kidogo cha damu. Vipimo vitakavyochukuliwa ni damu ya kioo kwa ajili ya vimelea vya malaria, tone la damu kwenye karatasi kwa vipimo vya kimolekuli na kiasi kidogo cha damu kwa ajili ya kipimo cha haraka cha malaria. Utaratibu huu wa kuchukuliwa kipimo hautakuwa wa kurudiwa bali utafanyika mara moja tu.

Athari

Ushiriki wako katika utafiti huu hauna athari ya aina yoyote mbali na kuhisi maumivu kidogo kwenye kidole wakati wa kutobolewa ili kuchukua damu na hayatadumu kwa muda mrefu. Kuna faida kadhaa za kushiriki kwako katika utafiti huu ikiwemo na kusaidia katika kufahamu njia mbadala zaidi za utafiti wa vimelea vya malaria. Vilevile utapata nafasi ya kutibiwa au kupewa rufaa/ushauri kwa tatizo la kiafya litakalogunduliwa wakati wa kuonana na daktari.

Motisha

Utapatiwa ushauri wa tiba bure na hakutakuwa na gharama yoyote kwa kushiriki kwako katika utafiti huu.

Usiri

Kwa muda wote wa utafiti huu, taarifa zako binafsi zitakusanywa na kuhifadhiwa kwa usiri. Taarifa zote hizi zitahifadhiwa kwa siri na uangalifu mkubwa wakati wa kufanya tathmini ya maelezo na vipimo vilivyokusanywa. Namba za siri na ufupisho

tu ndiyo utatumika na hizo taarifa hazitatolewa kwa mtu yeyote asiyehusika. Pamoja na hayo, uangalifu wa hali ya juu utakuwepo ili kutunza siri zote na hakuna taarifa zozote zenye kuonyesha majina ya wahusika katika utafiti huu zitakazotolewa kwenye machapisho au majarida ya kisayansi kuhusu utafiti huu.

Haki ya Kutoshiriki

Kushiriki kwako ni kwa hiari na una haki ya kutoshiriki bila pingamiza wala madhara yoyote mbeleni

Mawasiliano kama utakuwa na maswali yoyote

Utafiti huu umefanyiwa tathmini na kupata kibali cha tume ya Maadili ya Tiba ya Taasisi ya Taifa ya Utafiti wa Magonjwa ya Binadamu, Tanzania ambayo ni tume maalumu inayohakikisha kuwa washiriki wote wa utafiti wanakingwa na madhara yoyote yanayoweza kujitokeza.Endapo ungependa kupata maelezo zaidi kuhusiana na tume ya maadili ya tiba, wasiliana na: -

Professa Benezeth Mutayoba kwa simu ya kiganjani 0762411406 au ndugu Rehema Makoy: 0753653946. Pia unaweza wasiliana na Mwenyekiti wa kamati ya maadili ya tiba ya Taasisi ya Taifa ya utafiti wa magonjwa ya binadamu S. L. P 9653, Dar es Salaam, Tanzania, Simu: 022 21 30770.

Jina la Mhusika: ……

Sahihi ama dole gumba la Mhusika: …….

Tarehe: ……

Mahali: …….

Kama mhusika hajui kusoma na kuandika / mtoto. Jina na sahihi ya shahidi anayejua kusoma na kuandika au mzazi/mlezi (Inapobidi shahidi achaguliwe na mhusika): …….....

Sahihi /dole gumba…… Tarehe…….

Jina la Mtafiti: …….. Sahihi ya Mtafiti: ……

Tarehe…… Mahali ……..……