

UNIVERSITY OF GHANA  
COLLEGE OF HEALTH SCIENCES

**PREVALENCE OF PFHRP2 AND/OR PFHRP3 GENE  
DELETIONS IN *PLASMODIUM FALCIPARUM* ISOLATES  
AND THE PERFORMANCE OF HRP2 BASED MALARIA  
RAPID DIAGNOSTIC TESTS**

By

**EDITH BORTIER**

**(10325641)**

**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON,  
IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF  
MASTER OF SCIENCE IN MEDICAL LABORATORY SCIENCES DEGREE**

**DEPARTMENT OF MEDICAL LABORATORY SCIENCES**

**JULY 2019**

## DECLARATION

I, Edith Bortier, hereby declare that except for reference to other people's work, which I have duly cited, this thesis is the result of an original research work and that the material has not been presented either in whole or in part elsewhere for another degree and all experimental works were performed by me under the supervision of Dr Charles Brown.

---

**Edith Bortier**

**(Student)**

---

**Date**

---

**Dr Charles Brown**

**(Supervisor)**

---

**Date**

## ABSTRACT

**BACKGROUND:** Malaria rapid diagnostic tests (MRDTs) are important for malaria disease management. However, performance of the RDTs is affected when the targeted antigens in the parasite have a variation or are altogether absent. The most common parasite target antigen in RDTs, *Plasmodium falciparum* histidine-rich protein 2 (HRP2), has been reported to be absent in some *P. falciparum* parasites. Loss of the *pfhrp2* in *P. falciparum* parasites affects the accuracy of PfHRP2 based RDT kits when they are used in malaria diagnosis. Thus, to control malaria, determining where and how often *P. falciparum* parasites not having *pfhrp2* occur, is very important.

**AIM:** The aim was to investigate the prevalence of *pfhrp2* and/or *pfhrp3* gene deletions in *P. falciparum* isolates from southern Ghana and the performance of the currently used PfHRP2 based MRDTs.

**METHODS:** Samples were collected from sites in the southern part of Ghana: three Cocoa Clinics (Accra, Tafo, and Kumasi) and from three other health facilities, Ussher and Mamprobi polyclinics and 37 Military Hospital, all in Accra. Patients with febrile illness, referred by a clinician to the laboratories of these health facilities for a malaria test, were recruited. Blood samples for thick and thin blood smears, for malaria microscopy, and filter paper blots were obtained. All blood samples were tested using a HRP2-based malaria RDT. DNA was extracted from the dried filter paper blood blots using the TNES (Tris HCl, EDTA, NaCl, and SDS) buffer protocol. *Plasmodium falciparum* infection was confirmed by polymerase chain reaction (PCR). The presence of *pfhrp2* and *pfhrp3* genes was investigated by PCR.

**RESULTS:** A total of 371 patient samples, from Accra (58.5%), Kumasi (21.3%) and from Tafo (20.2%), were used in the study. PCR provided the highest number, 14.8% (55/371), of positive detections for *falciparum* infections. Microscopy detected parasites

in 20/261 (7.7%) samples and the minimum parasite density by microscopy was 430 parasites/ $\mu$ L. Out of the 371 samples, 27 (7.3%) were positive by RDT. The highest RDT positivity rate, 13.3% (10/75), was observed at Tafo. False negative RDT results were obtained in 43/55 (78.2%) of the negative branded RDT kits. Only two microscopy positive sample were RDT positive. Using 18SrDNA PCR, 55 (14.8%) samples were positive for *P. falciparum*. In Accra, 79.2% (19/24) of the PCR positive samples had *P. falciparum* parasites that lacked exon 2 of *pfhrp2*. In Tafo, on the other hand, only 7.4% (2/27) of the PCR positive samples had *P. falciparum* parasites that lacked exon 2 of *pfhrp2*. None of PCR positive samples had *P. falciparum* parasites that lacked exon 2 of *pfhrp2* in Kumasi. Only 33.3% (8/24) samples, all from Accra, lacked exon 2 of *pfhrp3*. In total, 38.1% (8/21) of the samples contained parasites that lacked exon 2 of both *pfhrp2* and *pfhrp3*. Fourteen negative- branded PfHRP2 RDT isolates, consisting of 13 (92.9%) samples from Accra and 1 (7.1%) from Tafo, were negative for the *pfhrp2* gene (*pfhrp2*-). Two samples, both negative- branded PfHRP2 RDT, were lacking the *pfhrp3* gene (*pfhrp3*-). Both samples were from Accra.

**CONCLUSION:** An overall RDT positivity rate of 7.3% (27/371) and false negative rate of 78.2% (43/55) was observed for the study sites. *Plasmodium falciparum* parasite populations with deletions of the *pfhrp2* and *pfhrp3* genes are present in the country. There is an urgent need for investigation of the prevalence and geographic distribution of these parasites.

## **DEDICATION**

This thesis is dedicated to my sons Jarren, Jason and Jadon Adams for all their time and patience for me to go through this course successfully.

## **ACKNOWLEDGEMENTS**

I first of all thank the Lord Almighty, in whom I renew my strength.

I wish to express my profound gratitude to my supervisor, Dr. Charles Brown for the support he provided and the knowledge he shared with me during each phase of this project. He was generous with resources as well as his patience and constructive criticisms.

My sincere gratitude to my husband and my mother who treasured my education and gave me the necessary support I needed.

My undying gratitude goes to Mr Prince Pappoe-Ashong of the Virology Department, School of Biomedical and Allied Health Sciences, for the technical support.

Lastly, to my very good friend late John Ayithey of blessed memory, course mates, and to all and sundry who in diverse ways have contributed to the success of my education, I say God richly bless you all. AMEN.

## TABLE OF CONTENTS

DECLARATION .....	ii
ABSTRACT.....	iii
DEDICATION.....	v
ACKNOWLEDGEMENTS.....	vi
TABLE OF CONTENTS.....	vii
LIST OF FIGURES .....	x
LIST OF TABLES.....	xi
LIST OF ABBREVIATIONS.....	xii
CHAPTER ONE.....	1
1.0 INTRODUCTION .....	1
1.1 BACKGROUND.....	1
1.2 PROBLEM STATEMENT .....	3
1.3 SIGNIFICANCE OF STUDY.....	3
1.4 AIM AND SPECIFIC OBJECTIVES.....	4
1.4.1 Aim.....	4
1.4.2 Specific Objectives.....	4
CHAPTER TWO .....	5
2.0 LITERATURE REVIEW .....	5
2.1 MALARIA .....	5
2.1.1 History .....	5
2.1.2 Aetiological Agents.....	6
2.1.2.1 <i>Plasmodium falciparum</i> .....	6
2.1.2.2 <i>Plasmodium vivax</i> .....	7
2.1.2.3 <i>Plasmodium ovale</i> .....	8
2.1.2.4 <i>Plasmodium malariae</i> .....	8
2.1.2.5 <i>Plasmodium knowlesi</i> .....	9
2.1.3 Transmission.....	9
2.1.3.1 Vectors .....	9
2.1.3.2 Mother to the growing foetus (congenital malaria) .....	10
2.1.3.3 Transfusion malaria .....	10
2.1.3.4 Needlestick injury .....	11
2.1.4 Pathogenesis .....	11



3.6.4 Confirmation of <i>P. falciparum</i> Infection by PCR .....	36
3.6.5 Detection of <i>pfhrp2</i> and <i>pfhrp3</i> Genes by PCR.....	36
3.6.6 Agarose Gel Electrophoresis .....	39
3.7 DATA ANALYSIS .....	39
3.8 ETHICS .....	41
CHAPTER FOUR.....	42
4.0 RESULTS .....	42
4.1 DETECTION OF <i>P. FALCIPARUM</i> MALARIA .....	42
4.1.1 Microscopy .....	42
4.1.2 RDT .....	42
4.1.3 PCR.....	44
4.2 PCR DETECTION OF <i>pfhrp2</i> AND <i>pfhrp3</i> .....	44
4.3 CONTRIBUTIONS OF <i>pfhrp2</i> and <i>pfhrp3</i> TO MALARIA DIAGNOSIS BY PfHRP-2 RDT .....	44
CHAPTER FIVE .....	46
5.0 DISCUSSION AND CONCLUSIONS .....	46
5.1 DISCUSSION .....	46
5.2 CONCLUSIONS.....	49
REFERENCES .....	51
APPENDIXES .....	59

## LIST OF FIGURES

<b>Figure 2.1</b>	Spatial distribution of all-age <i>Plasmodium falciparum</i> incidence in 2017	7
<b>Figure 2.2</b>	A malaria world map based on the estimated risk of malaria as defined by the US Centers for Disease Control and Prevention	14
<b>Figure 2.3</b>	Schematic drawing of a malaria rapid diagnostic test	18
<b>Figure 4.1</b>	Proportion of different detection methods positive for malaria	43
<b>Figure 4.2</b>	Occurrences of positivity (finding a positive test result) from -RDT in the samples obtained from the three sites	43
<b>Figure 4.3</b>	Ethidium bromide-stained 2.0% agarose gel electrophoregram of amplified <i>P. falciparum</i> 18S rRNA gene fragments using the primers rFAL1/rFAL2	45

## LIST OF TABLES

<b>Table 3.1</b>	Details of the oligonucleotide primers sequences used to amplify sequences of the <i>P. falciparum</i> gene for species identification	37
<b>Table 3.2a</b>	PCR reaction mixture for <i>P. falciparum</i> species identification	37
<b>Table 3.2b</b>	PCR thermocycling conditions for <i>P. falciparum</i> species identification	38
<b>Table 3.3</b>	Details of oligonucleotides primers for pfhrp2 and pfhrp3 genes PCR amplifications.	38
<b>Table 3.4a</b>	PCR reaction mixture for pfhrp2 and pfhrp3 genes PCR amplifications	40
<b>Table 3.4b</b>	PCR thermocycling conditions for pfhrp2 and pfhrp3 genes PCR amplifications.	40

## LIST OF ABBREVIATIONS

ACTs	Artemisinin-based combination therapies
APAD	Acetylpyridine adenine dinucleotide
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
FGR	Foetal growth restriction
GDH	Glutamate dehydrogenase
GICA	Gold immunochromatography assay
GMP	Good manufacturing practices
HRP	Histidine-rich protein
IFA	Immunofluorescence antibody
LAMP	Loop-mediated isothermal amplification
LDH	Lactate dehydrogenase
LDMS	Laser desorption mass spectrometry
MRDT	Malaria rapid diagnostic test
NMCP	National Malaria Control Programme
OPD	Out patient's department
PCR	Polymerase chain reaction
PFHRP	<i>Plasmodium falciparum</i> histidine-rich protein
PLDH	Plasmodium lactate dehydrogenase
QBC	Quantitative buffy coat
RDT	Rapid diagnostic test
rRNA	ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate

TAE	Tris acetate ethylene diamine tetraacetic acid
TNES	Tris, Sodium chloride, EDTA and SDS
TTM	Transfusion-transmitted malaria
tRNA	Transfer ribonucleic acid
WHO	World Health Organisation

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 BACKGROUND

Malaria is one of the life-threatening parasitic diseases transmitted to people through the bites of infected adult female *Anopheles* mosquitoes. It is caused by *Plasmodium* parasites (WHO, 2016b). Currently, five parasite species are known to human malaria, and 2 of these species – *P. falciparum* and *P. vivax* – present the highest threat. *Plasmodium falciparum*, which is the most common malaria parasite in Africa, causes most malaria-related deaths worldwide. *Plasmodium vivax*, on the other hand, is the main malaria parasite in countries outside sub-Saharan Africa (WHO, 2018).

According to the World Health Organisation (WHO) there was a decline in malaria-related mortality globally by 37% in 2016. Additionally, the African region persists in having an unduly high proportion of the global burden of malaria. In 2017, the region recorded 92% of malaria cases and 93% of malaria mortality (WHO, 2018). Globally, the WHO approach to malaria control efforts are based on vector control and improved diagnosis and treatment of patients with clinical malaria (WHO, 2011). However, to a very large extent, accurate and early diagnosis of malaria is the main determinant of the management and treatment of malaria.

Improving case management, as well as reducing malaria morbidity and mortality rates can be achieved by the use of malaria rapid diagnostic tests (MRDTs) (Maltha, Gillet, & Jacobs, 2013a; Mouatcho & Goldring, 2013). RDTs were employed in 75% of all diagnostic testing used to evaluate suspected malaria cases in Africa (WHO, 2018). In Ghana, and in all of Africa as well, RDTs are now first choice diagnostic tools for malaria diagnosis. Various malaria RDTs available capture at least four target antigens which

include *P. falciparum* histidine-rich protein 2 (HRP2) and lactate dehydrogenase (LDH), and the pan-plasmodial aldolase and LDH (Abba et al., 2014).

The most widely used RDT is PfHRP2. Histidine-rich protein 2 (HRP2), is an antigen specific to *P. falciparum*. Though it is highly abundant and heat stable, it is still detectable in the blood for up to a month after malaria parasites clearance (Kumar et al., 2012). In addition, PfHRP3, which is structurally similar to PfHRP2, has been shown to cross react with certain monoclonal antibodies directed against PfHRP2 (Lee et al., 2012). It has further been demonstrated that PfHRP2 based RDTs can produce false-positive results when the circulating HRP2 antigen persists even after antimalarial treatment, and false – negative results when parasitaemia levels fall below around 200 parasites/ $\mu$ L which is the detection limit for most RDTs which are commercially available (WHO, 2015b).

The performance of MRDTs to a large extent depends on the product quality, the conditions under which they are stored, parasite or operator factors and concentrations of parasite/antigen. One critical factor is the variations that can occur in the parasite antigens targeted by the RDTs such as presence or absence of the target epitopes and changes in the number of epitopes in a specific parasite isolate (Lee et al., 2012; Maltha et al., 2014). Genetic variability may be especially critical for RDTs based PfHRP2, as the majority of the RDTs make use of this antigen (Mouatcho & Goldring, 2013). Understanding the distribution and evolution of these mutant parasites is a priority for the WHO which recently hosted a technical consultation on *P. falciparum* hrp2/3 gene deletions and drafted interim guidance for investigating false negative RDT (WHO, 2017b). It is also unknown whether reliance on PfHRP2 based RDTs to guide treatment is exerting evolutionary pressure favouring the spread of this mutation. Monitoring the accurateness

of MRDT outcomes in addition to identifying issues affecting the diagnostic ability of MRDT is vital.

## **1.2 PROBLEM STATEMENT**

The RDTs mainly used in Ghana are based on PfHRP2. Recent studies reveal that the sensitivity of RDTs could be compromised due to genetic polymorphism of the parasite *pfhrp2* antigen causing false negative results. A variation or loss of the *pfhrp2* in *P. falciparum* parasites affects the accurateness of PfHRP2 based RDT kits when they are used in malaria diagnosis. Treatment of individuals with negative RDT results promotes drug resistance, wastes resources and can delay diagnosis of non-malaria causes of fever (WHO, 2017a, 2017b). In addition, in some instances, case management decisions will have to be revised.

The prevalence of parasites with the HRP-2 gene deletions may, however, vary in differing localities (Amir, Cheong, De Silva, & Lau, 2018). In a recent published data from Eritrea, the prevalence of *pfhrp2* deletions was estimated to be very high (80%), suggesting that an HRP2-only testing strategy may not be valid in some settings (Berhane et al., 2017). Very little data on the genetic variation of the *pfhrp2* is available in the country. Hence, the work aims to investigate the genetic variations of *pfhrp2* gene in malaria isolates from the southern part of Ghana.

## **1.3 SIGNIFICANCE OF STUDY**

Attributing false negative results to *pfhrp2/pfhrp3* deletion has significant implications for public health and thus following up on how accurately RDTs have performed is of utmost importance. Detecting the genetic variation of *pfhrp2* will define the frequency

and distribution of this diagnostically relevant mutation in circulating *P. falciparum* strains among symptomatic falciparum patients thus providing guidance in diagnosing and treatment. Results from the study can be helpful in determining the geographical locations of these gene deletions, establishing that RDT results that are negative are actually as a result gene deletion, and identifying locations where there may be need to change the strategies for diagnosis.

## **1.4 AIM AND SPECIFIC OBJECTIVES**

### **1.4.1 Aim**

The aim was to investigate the prevalence of pfhrp2 and/or pfhrp3 gene deletions in *P. falciparum* isolates from southern Ghana and the performance of the currently used PfHRP2 based MRDTs.

### **1.4.2 Specific Objectives**

The specific objectives were to:

1. Determine the prevalence of suspected false negative HRP2 RDT results among symptomatic patients with *P. falciparum* malaria.
2. Calculate the parasite density in patients with suspected false negative HRP2 RDT results.
3. Determine the prevalence of pfhrp2/3 gene deletions among symptomatic *P. falciparum* patients with a false negative HRP2 RDT result.
4. Determine the prevalence of pfhrp2/3 gene deletions causing false negative HRP2 RDTs among all symptomatic *P. falciparum* confirmed cases.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 MALARIA

##### 2.1.1 History

Malaria is an ancient disease. References to what was almost certainly malaria occur in a Chinese document from about 2700 BC, clay tablets from Mesopotamia from 2000 BC, Egyptian papyri from 1570 BC and Hindu texts as far back as the sixth century BC (Bruce-Chwatt, 1988). Moving into later centuries, the early Greeks, in particular, Homer (around 850 BC), Empedocles of Agrigentum (around 550 BC) and Hippocrates (around 400 BC), were thoroughly acquainted with the typical poor health, malarial fevers and enlarged spleens seen in people living in marshy places (Bruce-Chwatt, 1988).

The idea that malaria fevers were caused by miasmas rising from swamps persisted for over 2500 years. In addition, it was generally thought that the word malaria came from mal'aria an Italian word meaning "spoiled air" though this has been questioned. The search for the cause of malaria intensified following the development of the germ theory of infection by Louis Pasteur and Robert Koch in 1878-1879. However, scientific studies only became possible after the discovery of the parasites themselves by Charles Louis Alphonse Laveran in 1880 and the incrimination of mosquitoes as the vectors, first for avian malaria by Ronald Ross in 1897 and then for human malaria by the Italian scientists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava between 1898 and 1900 (Bruce-Chwatt, 1988; Poser & Bruyn, 1999).

Knowledge that malaria was caused by a protozoan parasite that invaded and multiplied in red blood cells was established by 1890. After a lot of confusion, three species with specific periodicities and other characteristics responsible for benign tertian (*Haemamoeba vivax*), malignant tertian (*Laverania malariae*) and quartan (*Haemamoeba malariae*) malaria now, respectively, *Plasmodium vivax*, *P. falciparum* and *P. malariae* were named (Stephens, 1922). In 1918, John Stephens, working in West Africa, discovered a fourth species which resembled *P. vivax* which he described as *P. ovale* in 1922 (Stephens, 1922).

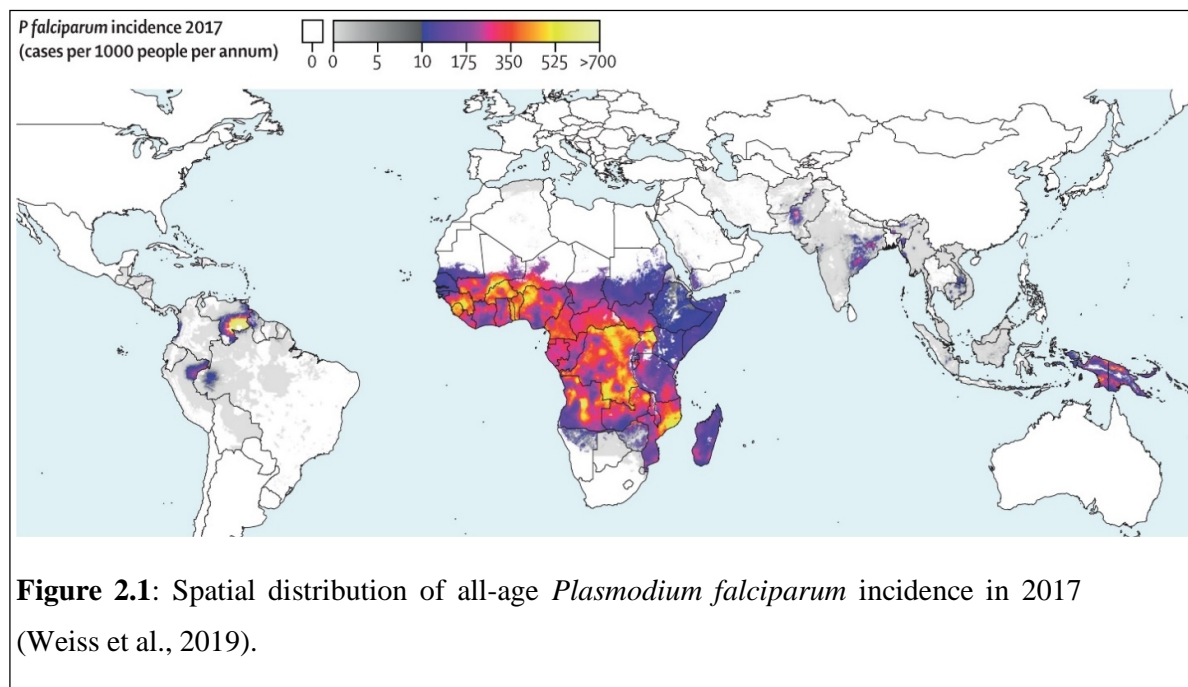
### **2.1.2 Aetiological Agents**

The aetiological agents of malaria are protozoa of the genus *Plasmodium*. There are five parasite species that cause malaria in humans, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and also by the simian parasite *P. knowlesi*. Two of these species – *P. falciparum* and *P. vivax* – pose the greatest threat (WHO, 2017c).

#### **2.1.2.1 *Plasmodium falciparum***

*Plasmodium falciparum* is found worldwide in tropical and subtropical areas, and especially in Africa where it predominates (Figure 2.1). *Plasmodium falciparum* can cause severe malaria because it multiplies rapidly in the blood, and can thus cause severe anaemia through haemolysis (WHO, 2019). In addition, the infected parasites can clog small blood vessels. When this occurs in the brain, cerebral malaria results, a complication that can be fatal.

*Plasmodium falciparum* is the most virulent strain of the malaria parasite species implicated in almost every malarial death among other malaria-causing species.



It causes most (99%) malaria-related deaths worldwide, and is the most prevalent malaria parasite on the African continent (WHO, 2019). In 2017, *P. falciparum* accounted for 99.7% of estimated malaria cases in the WHO African Region, as well as in the majority of cases in the WHO regions of South-East Asia (62.8%), the Eastern Mediterranean (69%) and the Western Pacific (71.9%) (WHO, 2019).

### 2.1.2.2 *Plasmodium vivax*

*Plasmodium vivax* is found mostly in Asia, Latin America, and in some parts of Africa. Because of the population densities especially in Asia it is probably the most prevalent human malaria parasite. *Plasmodium vivax* (as well as *P. ovale*) has dormant liver stages (hypnozoites) that can activate and invade the blood (relapse) several months or years after the infecting mosquito bite (WHO, 2015a).

Despite long being regarded as benign, evidence now shows that *P. vivax* is very often associated with severe, life-threatening, and fatal malaria in patients from endemic areas as well as in travellers (WHO, 2014).

### **2.1.2.3 *Plasmodium ovale***

*Plasmodium ovale* is found mainly in Africa (especially West Africa) and the islands of the western Pacific (Lysenko & Beljaev, 1969). It is biologically and morphologically very similar to *P. vivax*. However, in contrast to *P. vivax*, it is capable of infecting Duffy negative persons, who are mainly resident in sub-Saharan Africa. This is the reason for the higher prevalence of *P. ovale* (compared with *P. vivax*) in most of Africa (Collins & Jeffery, 2005).

Although it is commonly mentioned that *P. ovale* is very restricted in its occurrence being limited to West Africa (Faye, Konate, Rogier, & Trape, 1998), the Philippines, eastern Indonesia, and Papua New Guinea (Baird & Hoffman, 2004), it has been reported from Bangladesh (Fuehrer et al., 2010), Thailand (Cadigan & Desowitz, 1969) and Vietnam (Gleason, Fisher, Blumhardt, Roth, & Gaffney, 1970). However, the prevalence that has been reported is low (<5%) except from West Africa, where prevalences higher than 10% have been observed (Collins & Jeffery, 2005).

### **2.1.2.4 *Plasmodium malariae***

*Plasmodium malariae*, which is found worldwide, is the only human malaria parasite species that has a quartan cycle (three-day cycle) (Collins & Jeffery, 2007). The three other species have a tertian, two-day cycle. If untreated, *P. malariae* causes a long-lasting, chronic infection that in some cases can last a lifetime. In some chronically infected

patients *P. malariae* can cause serious complications such as the nephrotic syndrome (Collins & Jeffery, 2007).

#### **2.1.2.5 *Plasmodium knowlesi***

*Plasmodium knowlesi*, originally known to cause simian malaria, is now recognized as the fifth human malarial parasite (White, 2008). *Plasmodium knowlesi* occurs in all of Southeast Asia as a natural pathogen of long-tailed (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*). It has been shown to be an important cause of zoonotic malaria in that region, predominantly in Malaysia. *Plasmodium knowlesi* has a 24-hour replication cycle and so can rapidly progress from an uncomplicated to a severe infection; fatal cases have been reported (Cox-Singh et al., 2008). It has health, social and economic consequences for the regions affected by it (WHO, 2019)

### **2.1.3 Transmission**

#### **2.1.3.1 Vectors**

Principal mode of spread of malaria is by the bites of female *Anopheles* (=Gk., hurtful, harmful) mosquitoes. Of the more than “480 species of *Anopheles*, only about 50 species transmit malaria, with every continent having its own species of these mosquitoes: *An. Gambiae* complex in Africa, *An. Freeborni* in North America, and *An. culicifacies*, *An. fluviatilis*, *An. minimus*, *An. philippinensis*, *An. stephensi*, and *An. Sundaicus* in the Indian subcontinent” (Kiszewski et al., 2004).

*Anopheles* mosquitoes can be described as anthropophilic (having a preference for human blood meal), endophagic (indoor biters), and nocturnal (night biters) with highest biting activity at midnight, between 11 pm and 2 am (CDC, 2018). Mosquitoes locate their host

using visual, thermal (particularly skin temperature), and olfactory (particularly carbon dioxide, lactic acid) stimuli and moisture. Based on the intensity of these stimuli, individuals are differentially attracted, with grownups, males, and bigger persons preferentially more attractive (Barillas-Mury & Kumar, 2005).

During the bite of an infected person by a mosquito, the sexual forms of the parasite (gametocytes) are sucked up together with the blood. These gametocytes proceed with the sexual phase of the cycle inside the mosquito gut and the sporozoites that develop then fill the salivary glands of the infested mosquito. This female mosquito then inoculates the sporozoites into the blood stream of a new victim during a blood meal thereby spreading the infection (Barillas-Mury & Kumar, 2005).

#### **2.1.3.2 Mother to the growing foetus (congenital malaria)**

Malaria parasitized red cells can be transferred from an infected mother to the child through the placental or during child birth and this cause malaria in the new-born. This is known as congenital malaria. The occurrence of congenital malaria is most often not reported and has most of the time been thought of as being more prevalent in non-immune populations compared with endemic areas (Valecha, Bhatia, Mehta, Biswas, & Dash, 2007). Despite this, high prevalence rates (8% to 33%) for congenital malaria have been recorded from both non-immune populations (e.g. United States and Europe) and endemic ones (e.g. sub-Saharan Africa) (Valecha et al., 2007).

#### **2.1.3.3 Transfusion malaria**

Malaria can be transmitted by transfusion of blood from infected donors. This was first reported in 1911 (Valecha et al., 2007). Malaria can be efficiently transmitted by blood

transfusion from human to human and is undoubtedly responsible for the majority of transfusion-transmitted diseases in the world (Freimanis, Sedegah, Owusu-Ofori, Kumar, & Allain, 2013). The rate of transfusion-transmitted malaria (TTM) in malaria-endemic sub-Saharan regions is estimated to be between 14 and 28% (Freimanis et al., 2013). Several reports indicate that *P. falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi* and *P. ovale* can be transmitted either through blood donations or solid organ transplantations (Schindler et al., 2019).

#### **2.1.3.4 Needlestick injury**

Cases of malaria transmission through needlestick injuries, that have occurred by accident, among health care workers (some even fatal) or as a result of drug addicts sharing needles, have also been mentioned (Chauhan, Negi, Verma, & Thakur, 2009; Weir, 1997).

#### **2.1.4 Pathogenesis**

Malaria is an acute febrile illness. In a non-immune individual, symptoms usually appear 10-15 days after the infective mosquito bite. The first symptoms – fever (with temperatures rising above 37.5°C), headache, and chills – may be mild and difficult to recognize as malaria. If not treated within 24 hours, *falciparum* malaria can progress to severe illness and death (WHO, 2017c). Although the disease can manifest itself in mild or severe forms, individuals could also asymptotically harbour the parasites. In such individuals, their temperatures are usually below 37.5°C, and the levels of parasitaemia in their peripheral blood are at undetectable levels (WHO, 2012).

Malarial infection begins in an individual when a female *Anopheles* mosquito inoculates plasmodial sporozoites at the time of feeding. In most cases, relatively few sporozoites are injected (approx. 8–15), but up to 100 may be introduced in some instances. The sporozoites move to the liver, and undergo asexual reproduction over the next 7–10 days (Schofield & Hackett, 1993). No symptoms are seen during this phase. The parasites, which have now been transformed into merozoites, emerge from the hepatocytes in vesicles and move through the heart to the capillaries of the lungs. The merozoites, after the disintegration of the vesicles, are released and enter the bloodstream. In the bloodstream, they invade and multiply in erythrocytes, eventually rupture them, then the released merozoites rapidly reinvade other red cells and start a new asexual cycle. Only a sub-population of erythrocytes can be invaded, determined largely by red cell age. At this stage the host may still feel well, or may complain of vague non-specific symptoms. The clinical symptoms, including fever, thus occur in synchrony with the rupture of infected erythrocytes and the release of erythrocyte and parasite debris, including malarial pigment (haemozoin) and glycosphosphatidylinositol, the ‘malaria toxin’ (Boutlis, Riley, Anstey, & de Souza, 2005; Schofield & Hackett, 1993).

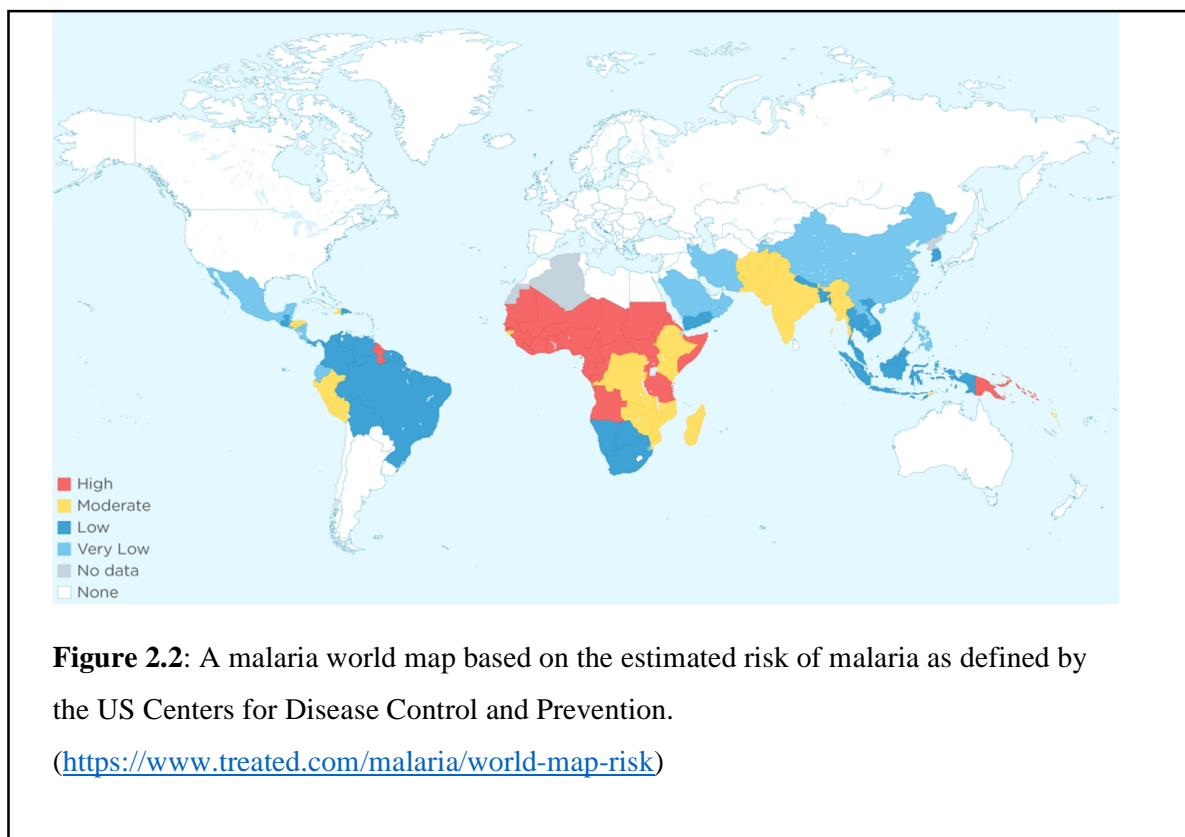
The associated symptoms of malaria include “severe anaemia, fever, thrombocytopenia, chills, headache, vomiting, muscle ache, anorexia, rigor, diarrhoea, abdominal discomfort, cough, seizures, respiratory distress, hypoglycaemia, metabolic acidosis, hyperlactaemia, coma associated with increased intracranial pressure (cerebral malaria), retinopathy, and complications of pregnancy, including preterm birth and low birth weight due to foetal growth restriction (FGR)” (WHO, 2019). As noted, the symptoms are associated with the rupture of the infected erythrocytes and the release of putative malaria toxins, which activate peripheral blood mononuclear cells and stimulate the

release of cytokines. The interplay between pro-inflammatory and anti-inflammatory cytokines, chemokines, growth factors, and effector molecules is believed to determine disease severity (Day et al., 1999; Kurtzhals et al., 1998).

### **2.1.5 Epidemiology**

Although malaria is of global health concern, it is sub-Saharan Africa that is disproportionately burdened with it. Malaria is one of the most important public health problems in terms of morbidity and mortality, causing more than 219 million cases and 435,000 deaths in 2017 (WHO, 2018). A total of 85 countries in the world are at risk of transmission of malaria infection (Figure 2.2). In 2017, a total of 200 million malaria cases (92%) occurred in the WHO African Region, followed by WHO South -East Asia Region (5%) and the WHO Eastern Mediterranean Region (2%). Of the total number of malaria deaths estimated in 2017; 93% occurred in the African Region (WHO, 2018).

Although the proportion of people exposed to malaria parasites has decreased during the last century, the absolute number of people at risk for malaria infection increased from 0.8 billion in 1990 to 3.3 billion in 2010, as a consequence of the absolute increase of the population living in malaria-endemic regions (Autino, Noris, Russo, & Castelli, 2012). The incidence rate of malaria also declined globally between 2010 and 2017, from 72 to 59 cases per 1000 population at risk. Although this represents an 18% reduction over the period, the number of cases per 1000 population at risk has stood at 59 for the past 3 years (WHO, 2018).



Over 35 million women living in malaria-endemic areas become pregnant each year (Dhiman et al., 2012). For these women, malaria is a threat both to themselves and to their babies. Malaria during pregnancy poses substantial risk to the mother, her foetus, and the neonate; the infection “contributes to as much as 15% of maternal anaemia, 14% of low birth weight infants, 30% of preventable low birth weight, 70% of intrauterine growth retardation, 36% of premature deliveries, and 8% of infant mortality” (Fried & Duffy, 2017). In areas of stable transmission where adult women have considerable acquired immunity, *P. falciparum* infection during pregnancy typically does not cause symptomatic malaria but may lead to maternal anaemia and placental malaria, especially among women having their first and second children (Fried & Duffy, 2017).

## **2.1.6 Laboratory Diagnosis**

### **2.1.6.1 Microscopic examination of blood smears**

Microscopic examination of Giemsa-stained blood smears has been the gold standard in the determination of malaria prevalence in epidemiologic surveys, providing a cheap means of quantifying and differentiating *Plasmodium* parasites (Moody, 2002). Using microscopy in areas of high malaria transmission, about 75% of the infections have been detected whereas in areas of low transmission, this technique has failed in the detection of up to 88% of infections (Okell, Ghani, Lyons, & Drakeley, 2009). Also, the level of expertise of malaria microscopists, equipment quality, and amount of work present may result in wrong parasite density estimates in addition to failure to accurately differentiate species (Wongsrichanalai, Barcus, Muth, Sutamihardja, & Wernsdorfer, 2007).

The procedure for microscopic examination is labour-intensive, and requires at least, one hour (usually more in a health centre with numerous suspected cases of malaria) from blood collection to results. Moreover, many health facilities in Africa do not possess a microscope, and even if there is a microscope in good working condition, well-trained and experienced microscopists are scarce. High-quality Giemsa stain and immersion oil may also be lacking in peripheral health centres. Within this context, rapid diagnostic tests (RDTs) are affordable alternative diagnostic tools in the absence of a microscope (Bisoffi et al., 2010). Thus, recent WHO guidelines recommended application of the universal “test and treat” strategy for malaria mainly by use of the RDT in all areas (WHO, 2015b, 2017b).

### **2.1.6.2 Quantitative buffy coat (QBC) technique**

The QBC technique was introduced in a bid to enhance microscopic detection of parasites and make malaria diagnosis more simple (Clendennen, Long, & Baird, 1995). It includes the staining of the parasites deoxyribonucleic acid (DNA) acid in micro-haematocrit tubes with fluorescent dyes, e.g. acridine orange, followed by detection by epi-fluorescent microscopy. A finger-prick blood is collected in a haematocrit tube containing acridine orange and anticoagulant. The tube is spun (12,000 g for 5) min and examined with an epi-fluorescent microscope (Chotivanich, Silamut, & Day, 2006).

Parasite nuclei fluoresce bright green, while cytoplasm appears yellow-orange. The QBC technique appears to be rapid and sensitive for diagnosing malaria in several laboratory settings (Adeoye & Nga, 2007; Bhandari, Raghuvver, Rajeev, & Bhandari, 2008). Even though it enhances sensitivity for *P. falciparum*, it reduces sensitivity for non-*falciparum* species and decreases specificity due to staining of leukocyte DNA (Moody, 2002). QBC technique is simple, reliable, and user friendly, but it requires specialized instrumentation, is more costly than conventional microscopy and is poor at determining species and numbers of parasites (Tangpukdee, Duangdee, Wilairatana, & Krudsood, 2009).

### **2.1.6.3 Malaria rapid diagnostic tests (MRDTs)**

RDTs for malaria were introduced in the 1990s and provided hope for a simple, accurate diagnostic test that could be carried out in health facilities in the absence of other diagnostic modalities. More recently, RDTs have been used as screening tools in field-based surveys since the results are ready within a short time thus allowing treatment on site (Endeshaw et al., 2008; Keating, Miller, Bennett, Moonga, & Eisele, 2009).

Malaria RDT (MRDT) kits are “designed to detect either *P. falciparum* specifically or discriminately detect both *P. falciparum* in addition to another human malaria parasite or indiscriminately detect all human malaria parasites” (WHO, 2011, 2015b). The main antigens that malaria RDT kits detect are pfhrp2, parasite lactate dehydrogenase (pLDH), and parasite aldolase (pAldo). The Pfhrp2 antigen is specific to *P. falciparum*, occurs in large quantities and is thermostable, and is the most used antigen in the design of MRDTs (Iqbal, Sher, & Rab, 2000; Kumar et al., 2012).

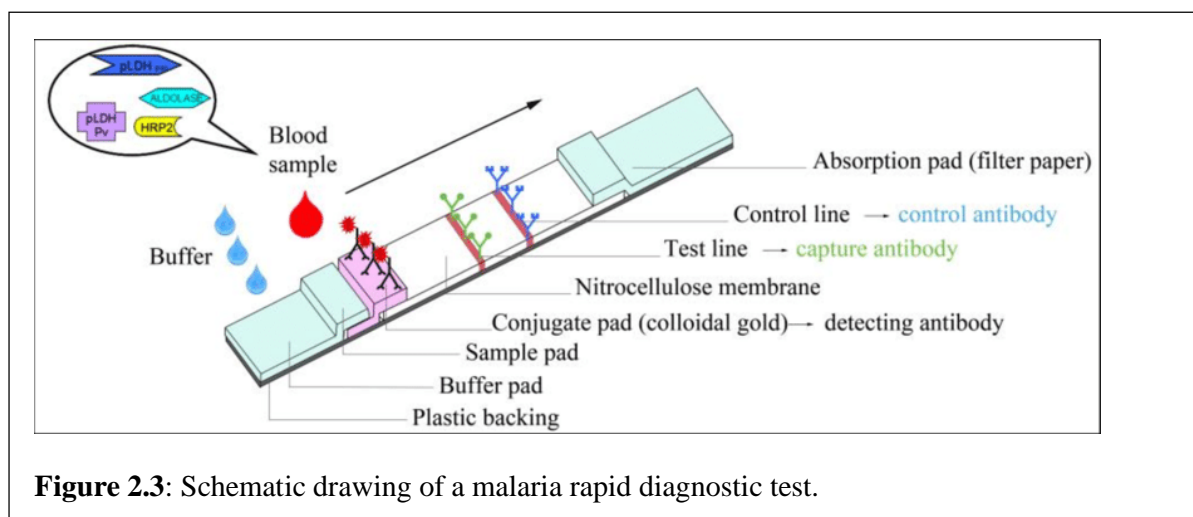
Field trials of histidine-rich protein-2 (HRP-2)-based RDTs showed sensitivity and specificity of over 90% for *P. falciparum* malaria at parasite densities of greater than 200 parasites/ $\mu$ L (Moody, 2002). In the malaria surveillance programmes in Tanzania, Gambia, Angola (2006–2007 malaria indicator survey) and Bangladesh, only RDTs were used in the monitoring of *Plasmodium* infections (Fançonny, Sebastião, Pires, Gamboa, & Nery, 2013; Haque et al., 2009). A sensitivity of 95.4% and specificity of 95.9% have been reported using RDTs performed by healthcare workers in a clinical trial in Tanzania (Reyburn et al., 2007).

According to the WHO, implementation of RDTs should be accompanied by a detailed quality control policy (WHO, 2011). To start with, RDTs should be bought from a manufacturer with good manufacturing practices (GMP). Secondly, tests should be carried out on each batch of RDTs that come into the country of use to certify that the kits have not been subjected to extreme temperature or other instances that may negatively impact the performance of the RDT. RDT performance is measured by testing known dilutions of parasites (typically 200 and 5,000 parasites/ $\mu$ L) and a negative control

(WHO, 2016a). In addition, the WHO suggests post-deployment testing at the health facility level, though these are less developed (WHO, 2016a).

### 2.1.6.3.1 Performance of *hrp-2*-based malaria rapid diagnostic tests (MRDTs)

Current MRDTs employ lateral flow immunochromatographic technology whereby clinical samples migrate as liquid across the surface of a nitrocellulose membrane by means of capillary action (Bell, Wilson, & Martin, 2005). Two sets of antibodies are used for a targeted parasite antigen – a capture antibody and a detection antibody (Figure 2.3). Either of these antibodies can be monoclonal or polyclonal. Monoclonal antibodies can be very specific but less sensitive, while polyclonal antibodies can be more sensitive but less specific. Even monoclonal antibodies designed to target different epitopes of the same antigen may exhibit quite different sensitivities and specificities (Lee et al., 2006).



The capture antibodies are sprayed as a stripe by machine onto the nitrocellulose membrane and bind to the membrane in an immobile phase. These fixed antibodies serve to extract and bind parasite antigen from the migrating liquid sample. The second set of

antibodies is conjugated to an indicator, typically gold particles, in a mobile phase. These antibody-indicator complexes bind to the parasite antigen that has been captured by the immobile antibody on the membrane, producing a visible line if the targeted antigen is present in the clinical sample (Bell et al., 2005).

The performance of MRDTs to a large extent depends on the product quality, the conditions under which they are stored, parasite or operator factors and concentrations of parasite/antigen. One critical factor is the variations that can occur in the parasite antigens targeted by the RDTs such as presence or absence of the target epitopes and changes in the number of epitopes in a specific parasite isolate (Maltha et al., 2014).

#### **2.1.6.4 Serological tests**

Immunofluorescence antibody testing (IFA) has been shown to be a dependable serologic test for malaria (She et al., 2007). Serological methods for malaria diagnosis are usually based on the detection of antibodies against asexual blood stage malaria parasites. Even though IFA is time-consuming and subjective, it is highly sensitive and specific (Sulzer, Wilson, & Hall, 1969).

IFA based on the principle that after infection, each *Plasmodium* species produces specific antibodies within a fortnight of the primary infection and these antibodies are still present even 3-6 months after parasite clearance. IFA uses specific antigen or crude antigen prepared on a slide, coated and kept at -30°C until used, and quantifies both IgG and IgM antibodies in patient serum samples (Chotivanich et al., 2006).

IFA is beneficial in field- based studies, screening of potential blood donors, and in some few cases for providing evidence of recent infection in non-immunes. Additionally, IFA cannot be automated and this limits the number of cases to be studied daily. It also requires fluorescence microscopy and trained technicians; readings can be influenced by the level of training of the technician, particularly for serum samples with low antibody titres. Moreover, the lack of IFA reagent standardization makes it impractical for routine use in blood transfusion centres, and for harmonizing inter-laboratory results. In conclusion, IFA is simple and sensitive, but time-consuming.

#### **2.1.6.5 Molecular diagnostic methods**

New laboratory diagnostic techniques that display high sensitivity and high specificity, without subjective variation, are urgently needed in various laboratories. Recent developments in molecular biological technologies, e.g. PCR, loop-mediated isothermal amplification (LAMP), microarray, mass spectrometry (MS), and flow cytometric (FCM) assay techniques, have permitted extensive characterisation of the malaria parasite and are generating new strategies for malaria diagnosis.

##### ***2.1.6.5.1 Polymerase chain reaction (PCR) technique***

PCR-based techniques in the molecular diagnosis of malaria have turned out to be one of the most specific and sensitive methods of diagnosis, especially in malaria cases presenting with low parasite levels or multiplicity of infection (Morassin, Fabre, Berry, & Magnaval, 2002). The PCR technique is widely used in the confirmation of malaria infection, follow-up of therapeutic response, and identification of drug resistance (Chotivanich et al, 2006). It was found to be more sensitive than QBC and some RDTs (Rakotonirina et al., 2008).

Concerning the gold standard method for malaria diagnosis, PCR has shown higher sensitivity and specificity than conventional microscopic examination of stained peripheral blood smears, and now seems the best method for malaria diagnosis (Morassin et al, 2002). PCR can detect as few as 1-5 parasites/ $\mu$ l of blood ( $\leq 0.0001\%$  of infected red blood cells) compared with around 50-100 parasites/ $\mu$ l of blood by microscopy or RDT (Britton, Cheng, & McCarthy, 2016). In addition, PCR is useful for detection of parasites that have become resistant to the anti-malaria drug, and in cases of multiple infections. Some PCR variations e.g., nested PCR, real-time PCR, and reverse transcription PCR, have provided reliable results where traditional diagnostic methods have given mixed or inconsistent results (Imwong et al., 2008).

The usage of PCR has some drawbacks, though the technique seems to have eliminated the two major difficulties of malaria diagnosis-sensitivity and specificity. The main obstacles to PCR use are the “complex methodologies” involved, its cost, and the requirement for specially trained technicians. PCR, thus, is not regularly employed in low-income countries because of the complexity of the testing and funds required to carry out these tests satisfactorily and regularly (Mens et al., 2007). Ensuring that results remain consistent and equipment are properly maintained are also very important for the PCR technique. Consequently, PCR may not be appropriate for malaria diagnosis in far-off rural places or even in daily clinical diagnostic settings (Baker et al., 2005; Hanscheid & Grobusch, 2002).

#### ***2..1.6.5.2 Loop-mediated isothermal amplification (LAMP) technique***

The LAMP technique is a straight forward and low-cost molecular malaria diagnostic test targeted at the conserved 18S ribosomal RNA gene of *P. falciparum* (Poon et al., 2006).

High sensitivity and specificity for *P. falciparum*, as well as *P. vivax*, *P. ovale* and *P. malariae* (Aonuma et al., 2008) and *P. knowlesi* (Piera et al., 2017) have been reported. LAMP appears to be easier, more sensitive, quicker and less expensive compared with PCR. In areas where vector-borne diseases, such as malaria, are prevalent, the LAMP technique can be more reliable and useful for routine screening.

#### **2.1.6.5.3 Microarray**

The principle of the microarray technique parallels traditional Southern hybridization. Labelled targets obtained from nucleic acids in the test sample are hybridized to probes on the array and this makes it possible to analyse several gene targets in one experiment. Ideally, this technique would be miniaturized and automated for point-of-care diagnostics (Holland & Kiechle, 2005). A pan-microbial oligonucleotide microarray has been developed for infectious disease diagnosis and has identified *P. falciparum* accurately in clinical specimens (Palacios et al., 2007).

#### **2.1.6.6 Flow cytometry analysis**

Reports of the use of flow cytometry for malaria diagnosis are available (Izumiyama, Omura, Takasaki, Ohmae, & Asahi, 2009). This technique detects hemozoin, a toxic but essential disposal by-product made when the malaria parasites digest haemoglobin in infected host red cells. Hemozoin within phagocytes can be detected by depolarization of laser light, as cells pass through a flow-cytometer channel. This method may provide a “sensitivity of 49-98%, and a specificity of 82-97%, for malarial diagnosis” (Grobusch et al., 2003) and is potentially useful for diagnosing clinically unsuspected malaria. However, it is labour intensive, requires trained technicians and expensive diagnostic

equipment, and other bacterial or viral infections may be detected leading to false positives.

#### **2.1.6.7 Mass spectrophotometry**

Mass spectrophotometry is an *in vitro* malaria parasite detection technique with a sensitivity of 10 parasites/ $\mu$ l of blood (Scholl et al., 2004). It comprises a protocol for clean-up of whole blood samples, followed by direct ultraviolet laser desorption mass spectrometry (LDMS). For malaria diagnosis, the principle of LDMS is to identify a parasite specific biomarker in clinical samples which in the case of malaria is the haem from haemozoin.

LDMS is automated and has a rapid turnaround time and high throughput capability. In comparison with using microscopy for malaria diagnosis which requires a highly trained technician and a minimum time of 30 minutes (maximum 1 hour) for the examination of a peripheral blood smear, LDMS analysis of a sample takes < 1 min (School et al, 2004). Nevertheless, in far off countryside places that lack electric power supply the conditions are inappropriate for high-tech mass spectrometers. Future developments in equipment and techniques should make this method more practicable.

## **2.2 PLASMODIUM FALCIPARUM ANTIGENS**

### **2.2.1 Histidine-rich protein 2 (HRP2)**

The histidine -rich protein 2 is a histidine and alanine rich, water –soluble protein, which is contained in several cell compartments including the parasite cytoplasm. Only *P. falciparum* trophozoites expressed this antigen (Beadle et al., 1994; Iqbal et al., 2000). HRP2 from *P. falciparum* has been “implicated in the biocrystallization of hemozoin, an

inert, crystalline from a ferriprotoporphyrin IX ( $\text{Fe}^{3+}$ )-PPIX) produced by the parasite". A large quantity of the HRP2 is released by the parasite into the bloodstream of the host and the antigen can be detected in red cells and some body fluids (e.g. cerebrospinal fluid) and even urine as a secreted water-soluble protein (Rock et al., 1987). The antigens can still be detected in the circulating blood after the parasitaemia has cleared or has been greatly reduced. Generally, 14 - 31 days elapse after treatment success before HRP2 based tests become negative which affects their potential usage for active infection detection (Humar, Ohrt, Harrington, Pillai, & Kain, 1997).

#### **2.2.1.1 *Plasmodium falciparum* histidine-rich protein-2 (PfHRP2) and reported genetic variations**

PfHRP2 has been sequenced and shown to contain 34% histidine and 37% alanine, as deduced from cloned genomic DNA (Wellems & Howard, 1986). Studies using a monoclonal antibody specifically directed against this protein have shown that PfHRP2 is synthesized throughout the erythrocytic stage of the parasite (Howard et al., 1986). PfHRP2 is produced exclusively by *P. falciparum* and exported out of the parasitophorous vacuole into the red blood cell (RBC). Upon RBC lysis, HRP-2 is released into the blood stream where it is found at concentrations  $> 1000\text{ng/mL}$ , and can be detected at least 1-month post clearance of parasites (Dondorp et al., 2005).

Furthermore, PfHRP2 is water-soluble, and is synthesized by both asexual and early sexual stages of the parasite. Its production increases as the asexual parasite matures such that most ( $\sim 90\%$ ) are released during schizogony (Desakorn et al., 2005). Circulating PfHRP2 may be unbound in plasma, antibody-bound in plasma, inside infected erythrocytes, bound to uninfected erythrocytes as part of immune complexes, and/or

bound to other cells such as leukocytes (Kifude et al., 2008). Its presence in plasma has allowed the development of inexpensive point-of-care RDTs (Moody, 2002).

A major concern with the use of PfHRP2-based RDTs for the diagnosis of malaria has been the reports of *P. falciparum* isolates lacking the *pfhrp2* and *pfhrp3* genes, which respectively encode the PfHRP2 protein and the related PfHRP3 protein (Gamboa et al., 2010; Koita et al., 2012; Kumar et al., 2012). Even though a number of studies have failed to fully corroborate these reports (Baker et al., 2010; Baker et al., 2005), the clinical significance of *hrp-2* gene sequence variation cannot be overemphasized.

Evaluations of two PfHRP2 detecting RDTs in Peru demonstrated sensitivity for *P. falciparum* diagnosis of 95% (Forney et al., 2003) and 53.5% (Bendezu et al., 2010). Consequently, Maltha et al. (2013a) assessed the diagnostic accuracy of a panel of different RDT products for malaria diagnosis in the Peruvian Amazon, with particular focus on the impact of *pfhrp2* and *pfhrp3* gene deletions on diagnostic sensitivity and of cross-reactions on diagnostic specificity. The study reported that “pLDH-detecting RDTs performed significantly better for *P. falciparum* diagnosis compared to PfHRP2-detecting RDTs in this geographical region”. The low sensitivity of PfHRP2-detecting RDTs was attributed to *pfhrp2* gene deletions which invariably led to false negative PfHRP2 results regardless of the parasite density. In the studies of Gamboa et al. (2010) and Maltha et al. (2013a), the prevalence of *P. falciparum* isolates lacking the *pfhrp2* gene were reported to be 41.0% and 25.7% respectively. In the latter study, the *pfhrp2* gene deletions were found at different sites, but not at all health centres, and all the isolates with the *pfhrp2* gene deletions were found in samples from symptomatic patients with high levels of parasitaemia. In Ghana, a malaria-endemic region where PfHRP-2-based RDTs are

widely used (sometimes, not in conjunction with microscopy), a study by Amoah, Abankwa, and Oppong (2016) reported a 33.3% prevalence of *pfhrp2* gene deletions among *P. falciparum* isolates originating from healthy children in two cities – Accra and Cape Coast.

The clinical implications of the *pfhrp2* deletions cited above and *pfhrp3* gene deletions are emphasized in a previous report by Houze, Hubert, Cohen, Rivetz, and Le Bras (2011). The authors reported a false negative RDT result in a French patient whose blood smear examination showed a parasitaemia of 144,000 parasites/ $\mu$ L, corresponding to 3.2% of infected erythrocytes. In this same patient, the Now ICT malaria test (Binax; Inverness Medical, France) showed positive results for the presence of the *pAldol* gene, but negative results for the presence of the *pfhrp2* gene, suggesting non-*P. falciparum* malaria. This observation, in addition to the fact that the patient had recently travelled to a *P. vivax*-endemic region (the Brazilian Amazon region), led to the diagnosis of *P. vivax* malaria, and the patient was treated with chloroquine. There was no clinical improvement after 2 days, and a second blood smear examination in the same laboratory showed a parasitaemia of 1,890,000 parasites/ $\mu$ L (corresponding to 42% of infected erythrocytes). The patient was then transferred to an expert malaria centre, where a new blood film examination was performed. The microscopic-stage pattern of parasites observed was specific to *P. falciparum*: old trophozoites with hemozoin and Maurer's clefts were predominant compared to ring-stage parasites. A repetition of the Now ICT malaria test (Binax; Inverness Medical, France) showed positive results for the presence of the *pAldolase* gene, but negative results for the presence of the *pfhrp2* gene. The patient, after treatment with quinine, fully recovered. Further investigations of the discordant RDT results using PCR targeting the *pfhrp2* and *pfhrp3* genes revealed, at least, partial deletion

of those genes, explaining the molecular basis for the observed discrepancy. It has been postulated that “p<sub>fh</sub>rp3 might compensate for absence of p<sub>fh</sub>rp2 in PfHRP2-detecting diagnosis, due to cross-reaction of PfHRP3 with PfHRP2 antibodies” (Baker et al., 2005; Gamboa et al., 2010).

### **2.2.2 *Plasmodium* lactate dehydrogenase (pLDH)**

*Plasmodium* lactate dehydrogenase (pLDH) is a key enzyme in the glycolytic pathway of *Plasmodium* species and has isomers specific to the species. This enzyme is secreted in the host’s peripheral blood and is detectable within 24 hours of effective malaria treatment (Harani et al., 2006). Due to these characteristics, pLDH has become a known reliable marker in detecting the presence of viable *Plasmodium* in the blood and is also widely used for screening in malaria-endemic countries (Lee et al., 2012). However, it must be noted that pLDH is only detectable in the presence of live parasites (Piper et al., 1999).

Because of the lack of a functional Krebs cycle, *Plasmodium* parasites heavily rely on LDH for survival during their intraerythrocytic stages and therefore, the only source of their adenosine triphosphate is through glycolysis together with fermentation. pLDH can be differentiated from mammalian LDH in both structure and kinetic features. In terms of structure, pLDH has a five-residue insertion (DKEWN) in their active site loop which activates during catalysis and closes down the active site. This insertion greatly enhances the substrate-specificity of pLDH compared to the human muscle and heart LDH isotopes. When it comes to kinetic feature, all pLDH differ from mammalian LDH by the susceptibility of the later to be inhibited by excess levels of the substrate pyruvate while pLDH exhibits decreased marked substrate inhibition. Also, pLDH has an ability to readily use the synthetic coenzyme 3-acetylpyridine adenine dinucleotide (APAD) as its

cofactor (Makler, Piper, & Milhous, 1998). Brown et al. (2004) compared the structure of the pLDH of the four human plasmodial species. They have reported a 90-92% structure similarity of the pLDH from *P. vivax*, *P. malariae* and *P. ovale* to pLDH from *P. falciparum*. However, there are significant differences between the *Plasmodium* species when it comes to kinetic properties and sensitivity to inhibitors. Since pLDH is an effective target for antibody-based malaria diagnosis by numerous researches, easy-to-operate RDTs that follow the principle of lateral flow immunochromatography had been developed.

### **2.2.3 Aldolase**

Another major enzyme involved in the glycolytic pathway of *Plasmodium* is aldolase, a homotetrameric protein which catalyses the cleavage of fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Each subunit of the enzyme has a molecular weight of approximately 40 kDa. Aldolase can be found in the host's blood when the enzyme is released during infection or in the parasite's cytoplasm in its soluble and active form (Dobeli et al., 1990; Knapp, Hundt, & Kupper, 1990).

*Plasmodium* aldolase can be distinguished from human isoenzyme by its possession of some unique nucleotide sequences (Tritten, Matile, Brun, & Wittlin, 2009). Both *P. falciparum* and *P. vivax* have only one aldolase isoenzyme and a great proportion of the amino acid sequences are greatly conserved in all *Plasmodium* species (Lee et al., 2006). In RDTs, aldolase is usually used as a pan-malaria antigen. Since *Plasmodium* aldolase is highly conserved during evolution, it makes the enzyme a target of choice when analysing isolates (Tritten et al., 2009). However, in a study by Bell et al. (2005), they have reported that in comparison to HRP2 based RDTs, aldolase and pLDH-based RDTs

are less sensitive due to the transient presence of the enzymes in the blood. A number of studies have also showed poor sensitivity of aldolase RDTs which called for further studies on the genetic diversity of aldolase. In contrast, a study by Lee et al. (2006) on the diversity in *P. falciparum* and *P. vivax* aldolase showed results that aldolase is not a cause of low RDT sensitivity. However, when it comes to detecting *P. vivax* infection, aldolase as a target antigen showed a more reliable diagnosis as reported by Dzakah et al. (2014) who assessed the relative performance of four RDTs that emphasized the detection of *P. vivax* antigens. Consequently, researchers have noted that a more sensitive assay for diagnosis of *P. vivax* infection can be developed combining aldolase and pLDH in RDTs (Dzakah et al, 2014).

## **2.7 PUBLIC HEALTH IMPLICATION OF *PFHRP2* AND *PFHRP* DELETIONS**

*Plasmodium falciparum* parasites with either a partial or complete deletion of the *pfhrp2* gene do not express the PfHRP2 antigen and hence detection by PfHRP2-based RDTs is compromised. Though these parasites might still have functioning *pfhrp3* genes encoding PfHRP3 protein, this protein is in low abundance and is less sensitive to *pfhrp2* detecting monoclonal antibodies. This means detection of an infection with such parasites may only be possible with PfHRP2-detecting RDTs at higher parasite densities (>1,000 parasites/ $\mu$ L) (Baker et al., 2005). It is therefore believed that a higher prevalence of parasites that lack *pfhrp2* gene will result in a higher rate of false negative results when using PfHRP2-based RDTs. This is particularly true for localities where there is low transmission and low incidence of multi-clone infection since parasites without PfHRP2 are less likely to co-infect with other strains with PfHRP2 in the same individual, and parasite densities in such cases are usually low.

False negative results will lead to problems in the management and control of malaria and this effect has been demonstrated in a study that compared sensitivities of several PfHRP2-based and pLDH-based RDTs for detecting *P. falciparum* infections in health centres around Iquitos (Maltha, Gillet, & Jacobs, 2013b) (Maltha, Gillet, & Jacobs, 2013b). The samples of study included 19 parasite isolates having deleted the pfhrp2 gene. Based on pfhrp2 detection the sensitivity of 10 products were evaluated to be approximately between 70 to 72%, with none of the products detecting any of the 19 isolates containing parasites without pfhrp2. However, 4 pLDH-detecting RDTs detected all 19 of these isolates, giving an average sensitivity of 97-99% (Maltha et al., 2012). It can be seen clearly that the sensitivity of PfHRP2-based RDTs have been compromised in the Amazon region and were no longer suitable for the detection of malaria or diagnosing *P. falciparum* infections both in Peru and for returning travellers from other regions affected by HRP2 deletions.

## **CHAPTER THREE**

### **3.0 METHODS**

#### **3.1 STUDY DESIGN**

A cross-sectional study design was used. This was a facility-based study in which patients reporting to the various cocoa clinics and other health facilities with fever and a referral to the laboratory for malaria test were enrolled after seeking their consent.

#### **3.2 STUDY SITES**

Samples were collected from sites in the southern part of Ghana: three Cocoa Clinics (Accra, Tafo, and Kumasi) and from three other health facilities, Ussher and Mamprobi polyclinics and 37 Military Hospital, all in Accra.

Cocoa Clinic is a quasi –government owned hospital. The Clinic was founded in 1973 as a small office Clinic (at COCOBOD Head office) to provide medical treatment to staff and dependants of Ghana Cocoa Board and its Subsidiaries, Divisions and Departments. The Clinic has blossomed into a full-fledged medical facility catering for over 100 corporate bodies, private individuals, and health insurance scheme clients. Cocoa Clinic is one of the high-profile health facilities in the country with branches in strategic parts of the country.

Cocoa Clinic located closely to the entrance of the Cocoa Research Institute of Ghana. Accra Cocoa Clinic is located at Bubuashie Roundabout, Kaneshie, Accra. The Kumasi clinic is about 1 km away from the COCOBOD Jubilee House and a walking distance from the State Transport bus terminal. Attendance at these clinics' laboratory is highest

in Accra and Kumasi with an average monthly malaria record of 3097 and 2211, respectively. Tafo Cocoa Clinic has an average of 845 of malaria cases monthly.

Both Ussher and Mamprobi polyclinics are government owned and are located in James town and Mamprobi, respectively, in the Accra metropolitan district of the greater Accra region. Ussher polyclinic is located directly east of the Korle Lagoon whilst Mamprobi polyclinic is located south of Laterbiokorshie, northeast of old Dansoman and north of Chorkor.

The 37 Military Hospital is a specialist hospital located in Accra, on the main road between the Kotoka International Airport and central Accra. It is the largest military health facility in the republic of Ghana.

### 3.3 SAMPLE SIZE CALCULATION

Sample size was centred on the need to obtain relatively precise estimates of false negative HRP2 RDT outcomes due to *pfhrp2/3* deletions at each study site. Estimates for sample size were considered for obtaining proportions as a result of simple random sampling, using a sampling design effect ( $deft$ ) = 1.5 (to account for observations correlated within clinics vis-à-vis *pfhrp2/3* deletions) and a probability of committing a type-1 error = 95% (1-sided test), such that the 95% confidence interval does not overlap with the threshold of 5%. Thus:

$$n \geq deft \left[ \frac{Z^2(P)(1 - P)}{D^2} \right]$$

$$n \geq deft \left[ \frac{1.96^2(0.33)(1 - 0.33)}{0.05^2} \right]$$

$$n \geq 57.6$$

Where:

Z = z-score of the confidence level (95%) = 1.96

P = expected population prevalence of pfhrp2/3 gene deletions = 3.9% (Amoah et al., 2016)

d = margin of error = 5% = 0.05

As a minimum, therefore, a sample of 58 individuals with a *P. falciparum* infection per site was collected.

### **3.4 STUDY POPULATION**

Blood samples were collected from a patient who came to the selected health facilities showing symptoms consistent with uncomplicated malaria and had a referral to the outpatient laboratory for blood smears in line with national malaria treatment guidelines.

#### **3.4.1 Inclusion Criteria**

The inclusion criteria for blood sample collection was positive identification of *P. falciparum* mono-infection by microscopy in the blood smears of symptomatic patients over the age of 5 years.

#### **3.4.2 Exclusion Criteria**

Blood samples from patients diagnosed as having severe malaria or other conditions requiring urgent diagnosis or treatment and pregnant women were excluded from the study.

### **3.5 SAMPLE COLLECTION**

At the time of enrolment, demographic and clinical information was collected and a finger-prick blood sample was obtained for thick and thin blood smears, an RDT. In addition, filter paper dried blood spots (DBS) were prepared, air dried and stored in plastic transparent zip lock bags with desiccant for DNA extraction.

### **3.6 LABORATORY PROCEDURES**

#### **3.6.1 Microscopy**

Thick and thin blood smears were prepared on the same slide for each blood sample after the analysis of RDT. Two drops of blood with about 1 cm distance apart were placed on a clean labelled glass slide. For the thick blood film smear, the blood spot was stirred in circular motion with the corner edge of another slide. The thin blood film was prepared by placing the smooth edge of the spreader slide on the drop of blood at an angle of 45° and quickly smeared forward on the slide surface. The blood smears were allowed to air dry and thin film fixed with methanol. The slides were then stained with 10% Giemsa for 15 minutes after which the stain was washed off and air dried. Slides were examined using light microscope 100X oil immersion and 100 fields were scanned before a particular smear was declared negative. Parasitaemia was calculated by counting the number of parasites observed per 200 leukocytes, and assuming a total of 8,000 leukocytes per microliter.

#### **3.6.2 Rapid Diagnostic Tests**

Approximately 2 ml of whole blood was taken from consented patients into EDTA tubes by standard technique. For each sample, PFHRP2 based RDT was done using SD BIOLINE malaria Ag Pf cassettes. SD Bioline provided disposable specimen loop (5 ul)

was used to sample blood from the EDTA tubes unto RDT cassette sample well after which four drops of assay diluent was dispensed unto the assay diluent well and results read at a minimum time of 15 minutes or a maximum time of 30 minutes.

All blood samples were tested using HRP2-based malaria RDT. The RDT assays were performed and the results interpreted following the instructions provided by the manufacturers. The used RDT cassettes were kept at room temperature (25°C) for a maximum of 1 week, then their membranes were processed for extraction of DNA.

### **3.6.3 Extraction of Parasite DNA**

Genomic DNA was extracted from the membranes of the previously used PfHRP2 RDT kits. Briefly, the RDT cassette was opened and portions between the filter paper through to the nitrocellulose membrane and some of the conjugated pad were cut and placed into a 1.5 ml microcentrifuge; a separate scalpel was used for each RDT. For lysis, 200 µl TNES digestion buffer (10 mM Tris-HCl (pH 7.5), 400 mM NaCl, 100 mM EDTA, 0.60% SDS) was added to the microcentrifuge tube, followed by the addition of 6 µl of proteinase K (10 mg/ml) and incubation overnight in a heat block (Thermo Block TDB-120, Warren, United States of America) at 55°C. Samples were retrieved, 200 µl 5 M NaCl was added to each tube and vortexed briefly. The contents were then spun at 15000 rpm for 10 minutes. The supernatants were transferred into 1.5 ml Eppendorf tubes, and 800 µl 100% ethanol added to each tube and rocked gently back and forth.

Deoxyribonucleic acid (DNA) precipitation was visible at this moment. Samples were stored in a -21°C freezer for 3 hours. They were retrieved, allowed to thaw, and spun down at 14000 rpm for 30 minutes. The absolute ethanol was carefully poured off the

pellet, 500  $\mu$ l 70% ethanol added and the tubes spun again at 14000 rpm for 5 minutes. The 70% ethanol was poured off after spinning, tubes were blotted and air dried. The formed pellets were finally re-suspended in 200  $\mu$ l TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0.) and stored at -21°C.

#### **3.6.4 Confirmation of *P. falciparum* Infection by PCR.**

The nested PCR-based method for the human *Plasmodium* species identification was used (Singh et al., 1999). The initial PCR involved the use of the genus-specific oligonucleotide primer pair, rPLU5 and rPLU6, to amplify DNA fragment or sequence common to malaria species. The PCR product obtained was used as a template for the nested PCR using species-specific oligonucleotide primer pair rFAL1 and rFAL2 for *P. falciparum*. Sequences and other details of the primers used for the PCR are in table 3.1. The PCR reaction mix and conditions were set up as shown in tables 3.2a and 3.2b, respectively. Each PCR reaction was mixed thoroughly. The PCR reactions were carried out in a SEEAMP SCE1000 thermal cycler (Seegene Inc., Korea). *Plasmodium falciparum* 3D7 strain DNA was used as positive control and double-distilled water instead of DNA was used as negative control in all the PCR reactions carried out.

#### **3.6.5 Detection of pfhrp2 and pfhrp3 Genes by PCR**

The PCR amplifications were adapted from (Baker et al., 2005) with some minor modifications. All PCR amplifications were either nested or semi nested. Sequences and other details of the primers used for the PCR are in table 3.3.

**Table 3.1:** Details of the oligonucleotide primers sequences used to amplify sequences of the *P. falciparum* gene for species identification (Singh et al., 1999).

Purpose	Primer	Sequence (5'-3')	Diagnostic band size (bp)
<i>Plasmodium</i> sp.	rPLU5	CTTGTTGTTGCCTTAAACTTC	1200
	rPLU6	TTAAAATTGTTGCAGTTAAAACG	
<i>P. falciparum</i>	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	205
	rFAL2	ACACAATGAACTCAATCATGACTACCCGTC	

**Table 3.2a:** PCR reaction mixture for *P. falciparum* species identification.

Reagents	Volume per sample (µl)	Final concentration
	<b>1x</b>	
OneTaq® Quick-Load® 2X Master Mix with Standard Buffer*	6	1x
10 mM rPLU5 <sup>a</sup> / rFAL1 <sup>b</sup>	0.3	0.25 mM
10 mM rPLU6 <sup>a</sup> / rFAL2 <sup>b</sup>	0.3	0.25 mM
DNA template	5	
Nuclease free water	3.4	
<b>Total</b>	<b>12.0</b>	

\*New England Biolabs Inc. (NEB) [Ipswich, MA, USA]; <sup>a</sup>Nest 1 PCR; <sup>b</sup>Nest 2 PCR

**Table 3.2b:** PCR thermocycling conditions for *P. falciparum* species identification.

Cycle	Temperature	Time	No. of cycles
Initial Denaturation	95°C	5 minutes	1
Denaturation	94°C	1 minute	
Annealing	58°C	1 minutes	25 <sup>a</sup> / 30 <sup>b</sup>
Extension	68°C	2 minutes	
Final extension	68°C	10 minutes	1

<sup>a</sup>Nest 1 PCR; <sup>b</sup>Nest 2 PCR

**Table 3.3:** Details of oligonucleotides primers for pfhrp2 and pfhrp3 genes PCR amplifications.

Purpose	Primer	Sequence (5'-3')
<i>Pfhrp2</i> <sup>2</sup>	<b>Pfhrp2-F1</b>	CAAAAGGACTTAATTTAAATAAGAG- 3
	<b>Pfhrp2-R1</b>	AATAAATTTAATGGCGTAGGCA
	<b>Pfhrp2-F2</b>	ATTATTACACGAAACTCAAGCAC
<i>Pfhrp3</i> <sup>2</sup>	<b>Pfhrp3-F1</b>	AATGCAAAAGGACTTAATTC
	<b>Pfhrp3-R1</b>	TGGTGTAAGTGATGCGTAGT
	<b>Pfhrp3-F2</b>	AAATAAGAGATTATTACACGAAAG

The PCR reaction mix and conditions were set up as shown in tables 3.4a and 3.4b, respectively. Each PCR reaction was mixed thoroughly. The PCR reactions were carried out in a SEEAMP SCE1000 thermal cycler (Seegene Inc., Korea). Genomic DNA 3D7 (wild type) was used as control for the PCR amplifications. PCR amplification for all samples that give a negative result for any primer set was repeated using twice the volume of gDNA as template.

### **3.6.6 Agarose Gel Electrophoresis**

After the PCR, 10  $\mu$ l of PCR product was added to 2  $\mu$ l of 6x bromophenol blue loading dye and electrophoresed in 2% agarose gels stained with 0.5  $\mu$ g/ml ethidium bromide. The gels were prepared and run in 1x TAE buffer at 110V for 30-45 minutes and were observed and photographed over UV transilluminator at short wavelength using a Kodak EDAS 290 gel documentation system. The sizes of the PCR products were estimated by comparing with the mobility of a standard 100bp DNA ladder (New England Biolabs Inc., Ipswich, MA, USA).

### **3.7 DATA ANALYSIS**

Crosstab descriptive analysis was performed using IBM SPSS Statistics (version 24). Microsoft Excel was used to draw the graphs.

**Table 3.4a:** PCR reaction mixture for pfhrp2 and pfhrp3 genes PCR amplifications.

Reagents	Volume per sample (µl) 1x	Final concentration
OneTaq <sup>®</sup> Quick-Load <sup>®</sup> 2X Master Mix with Standard Buffer*	6	1x
10 mM Pfhrp2-F1 <sup>a</sup> /Pfhrp2- F2 <sup>b</sup> / Pfhrp3-F1 <sup>c</sup> / Pfhrp3- F2 <sup>d</sup>	0.3	0.25 mM
10 mM Pfhrp2-R1 <sup>e</sup> / Pfhrp3- R1 <sup>f</sup>	0.3	0.25 mM
DNA template	5	
Nuclease free water	3.4	
<b>Total</b>	<b>12.0</b>	

\*New England Biolabs Inc. (NEB) [Ipswich, MA, USA]; <sup>a,e</sup>Pfhrp2 nest 1 PCR; <sup>b,e</sup>Pfhrp2 nest 2 PCR; <sup>c,f</sup>Pfhrp3 nest 1 PCR; <sup>d,f</sup>Pfhr3 nest 2 PCR.

**Table 3.4b:** PCR thermocycling conditions for pfhrp2 and pfhrp3 genes PCR amplifications.

Cycle	Temperature	Time	No. of cycles
Initial Denaturation	95°C	10 minutes	1
Denaturation	95°C	50 seconds	
Annealing	55°C <sup>a</sup> / 51°C <sup>b</sup>	50 seconds	41
Extension	68°C	1 minute	
Final extension	68°C	5 minutes	1

<sup>a</sup>pfhrp2 PCR; <sup>b</sup>pfhrp3 PCR

### **3.8 ETHICS**

Ethical approval was obtained from the Ethics and Protocol Review Committee of the School of the College of Health Sciences (CHS), University of Ghana. Approval was also be sought from the management of the health facilities involved in the study as well from the Regional Health Directorate of Greater Accra Region. Before enrolment, all participants had the study explained to them following which written informed consent was obtained. For children between 12 years and 17 years, child assent was obtained while parental consent was obtained from parents and guardians of all children as well.

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.1 DETECTION OF *P. FALCIPARUM* MALARIA

A total of 371 patient samples, 217 (58.5%) from Accra, 79 (21.3%) from Kumasi, and 75 (20.2%) from Tafo, were used in the study. PCR provided the highest number, 14.8% (55/371) of positive detections (Figure 4.1) for *P. falciparum*.

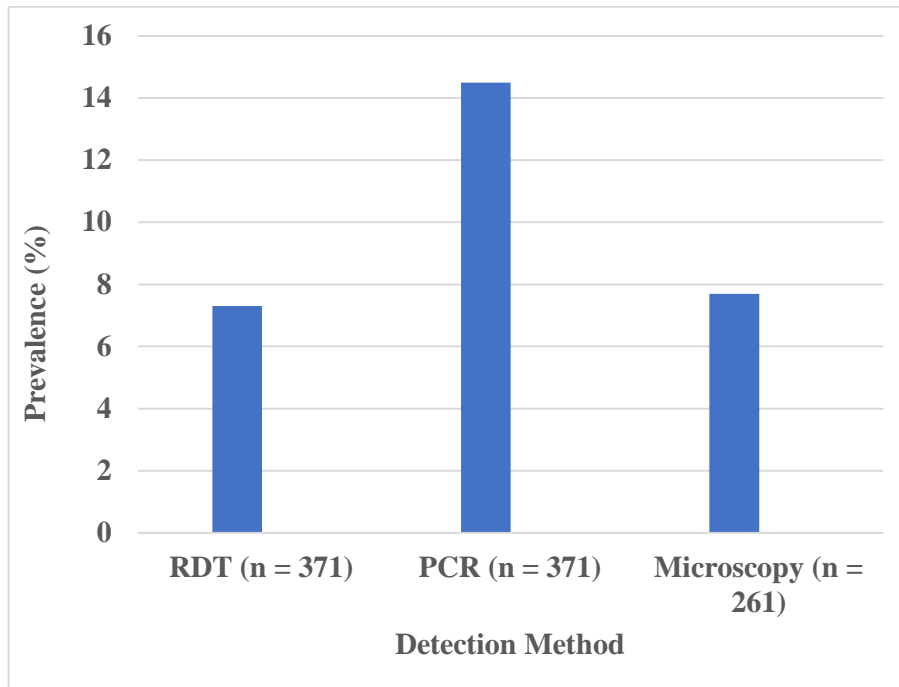
##### 4.1.1 Microscopy

Microscopy detected parasites in 20/261 (7.7%) samples. The 20 positive samples were obtained in Accra (45.0%, n = 9), Kumasi (20.0%, n = 4) and Tafo (35.0%, n = 7). Only two microscopy positive sample were RDT positive. The minimum parasite density reported by microscopy was 430 parasites/ $\mu$ L.

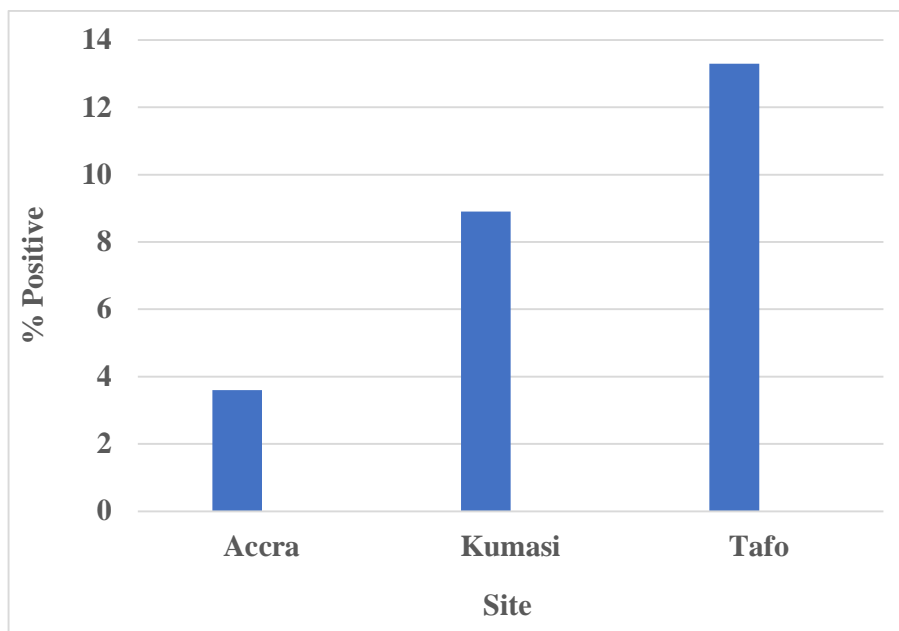
##### 4.1.2 RDT

Out of the 371 samples, 27 (7.3%) were positive by RDT. Of these 27 RDT positive samples, 10 (37.0%), 7 (26.0%) and 10 (37.0%), were obtained from Accra, Kumasi and Tafo, respectively. The highest RDT positivity rate, 13.3% (10/75), was observed at Tafo (Figure 4.2).

False negative RDT results were obtained in 43/55 (78.2%) of the negative branded RDT kits. Twenty-two (51.2%) of the false negative RDT results were obtained from Tafo, 18 (41.9%) from Accra and 3 (6.9%) from Kumasi.



**Figure 4.1:** Proportion of different detection methods positive for malaria.



**Figure 4.2:** The frequencies of positivity (obtaining a positive test results) obtained by - RDT in the samples collected from the three sites.

#### **4.1.3 PCR**

Using 18SrDNA PCR, 55 (14.8%) samples were positive for *P. falciparum* (Figure 4.2). Of these 55 PCR positive samples, 24 (43.6%), 4 (7.3%) and 27 (49.1%), were obtained from Accra, Kumasi and Tafo, respectively. For each of the *P. falciparum* PCR positive samples, the expected 205 bp fragment of the 18S rRNA gene was seen (Fig. 4.3).

#### **4.2 PCR DETECTION OF *pfhrp2* AND *pfhrp3***

In Accra, 79.2% (19/24) of the PCR positive samples had *P. falciparum* parasites that lacked exon 2 of *pfhrp2*. In Tafo, on the other hand, only 7.4% (2/27) of the PCR positive samples had *P. falciparum* parasites that lacked exon 2 of *pfhrp2*. None of PCR positive samples had *P. falciparum* parasites that lacked exon 2 of *pfhrp2* in Kumasi.

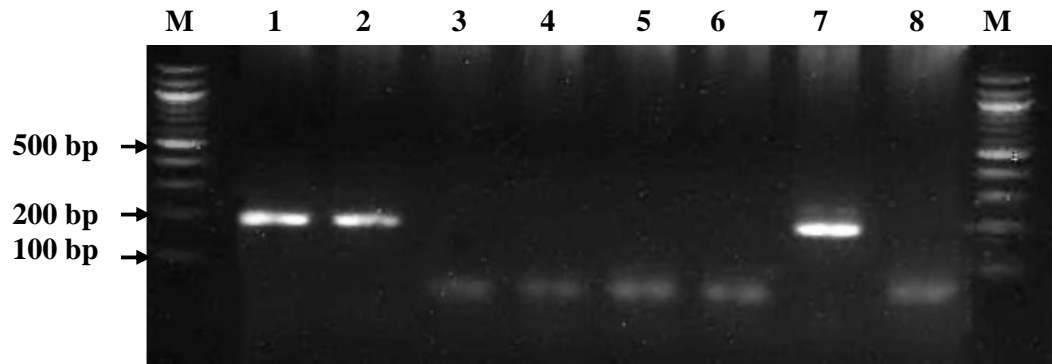
Only 33.3% (8/24) samples, all from Accra, lacked exon 2 of *pfhrp3*. In total, 38.1% (8/21) of the samples contained parasites that lacked exon 2 of both *pfhrp2* and *pfhrp3*.

#### **4.3 CONTRIBUTIONS OF *pfhrp2* and *pfhrp3* TO MALARIA DIAGNOSIS BY**

##### **PfHRP-2 RDT**

When genomic DNA from positive and negative branded PfHRP2 RDT kits, confirmed to carry *P. falciparum* parasite by PCR genotyping, were subjected to *pfhrp2* and *pfhrp3* gene PCR amplification, fourteen negative- branded PfHRP2 RDT isolates, consisting of 13 (92.9%) samples from Accra and 1 (7.1%) from Tafo, were negative for the *pfhrp2* gene (*pfhrp2*-).

Two samples, both negative- branded PfHRP2 RDT, were lacking the *pfhrp3* gene (*pfhrp3*-). Both samples were from Accra.



**Figure 4.3:** Ethidium bromide-stained 2.0% agarose gel electrophoregram of amplified *P. falciparum* 18S rRNA gene fragments using the primers rFAL1/rFAL2.

Lane M = 100 bp DNA Ladder (NEB); Lanes 1 and 2 = Samples positive for *P. falciparum* infection; Lanes 3-6 = Samples negative for *P. falciparum* infection; Lane 7 = positive control; Lane 8 = negative control

## CHAPTER FIVE

### 5.0 DISCUSSION AND CONCLUSIONS

#### 5.1 DISCUSSION

At present, PfHRP2 based RDTs are central to malaria control programmes, besides they allow malaria diagnosis even in the absence of a microscope. However, continued usage of these tests in areas of high malaria transmission is under threat by parasites that do not express PfHRP2 (Gupta et al., 2017; WHO, 2017a). Due to the fact that majority of the RDTs sold to the public target PfHRP2 expressed exclusively by *P. falciparum*, *pfhrp2/3* genetic diversity analysis of is of great interest to public health.

Until quite recently, the relatively few earlier studies on the PfHRP-2 based RDT kits in Ghana concentrated on finding the sensitivity and specificity of Pf-HRP2 RDT kits (Baiden et al., 2012; Nkrumah et al., 2011). A study by Amoah et al. (2016) conducted in Accra and Cape Coast provided further evidence for the existence of parasites carrying *pfhrp2*– in addition to how these parasites affect the accuracy of malaria diagnosis by RDTs based on this antigen in Ghana. The current study adds to those findings by analysing samples collected from Accra, in addition to Tafo and Kumasi.

Ghana is hyperendemic to malaria, thus RDTs are used for malaria diagnosis on a daily basis and throughout the year. Though microscopy is the gold standard for malaria diagnosis, it not performed to confirm the absence of parasitaemia in most hospital or clinic laboratories. What rather happens is that when RDTs are negative, it is assumed that the patient is not having malaria. As a consequence of this, it is critical that malaria diagnosis using RDT kits have high accuracy as this is indispensable if malaria is to be controlled (Bisoffi et al., 2010; Rakotonirina et al., 2008).

The overall RDT positivity rate of 7.3% (27/371) was relatively lower as compared to those of other studies carried out in the country (Amoah et al., 2016). The highest RDT positivity rate of 13.3% (10/75) was obtained at Tafo which although is a populous settlement in the country has a population density far lower than both Accra and Kumasi, the other two sites of the study. Compared with PCR, diagnosis of malaria infection by both microscopy and RDT were found to be lower which was in agreement with other studies carried out in other places (Berzosa et al., 2018; Gatti et al., 2007; Tham, Lee, Tan, Ting, & Kara, 1999). However, the quality of malaria diagnosis is imperative in all situations, as misdiagnosis can result in substantial morbidity and mortality.

A false negative RDT result was indicated when PCR detected the presence of malaria parasites although the RDT kit test result was negative for a particular sample. In this study, false negative RDT results were obtained in 43/55 (78.2%) of the negative branded RDT kits and this was higher than previously detected by Amoah et al. (2016). Because malaria is endemic in this country, this can have serious aftermaths in the study areas in the absence of any confirmatory diagnostic tests, such as PCR, for such negative RDT results (Gardner et al., 2002). False negative RDT results delay malaria treatment, potentially endangering life, in addition to the fact that the patient will serve as a source for uninterrupted transmission of malaria. Factors other than *pfhrp2* deletion or variation, such as very low parasite density or concentration of the target antigen and hyperparasitaemia might have accounted for the false negative RDT results (WHO, 2017a).

The findings from this study confirm that *P. falciparum* parasite populations with deletions of the *pfhrp2* and *pfhrp3* genes are present in the country. The deletions of the *pfhrp2* and *pfhrp3* genes were seen in Accra as well as Tafo but not in Kumasi. The samples from Kumasi also showed the lowest RDT negative results (6.9%). Thus, the absence of the deletions in the samples from Kumasi was not surprising though considering the fact that it a populous city similar and outcomes to that in Accra were expected.

The deletions of the *pfhrp2* and *pfhrp3* genes agrees with the study in 2016 by Amoah et al. (2016). In this study, *pfhrp2* deletions were more prevalent than *pfhrp3* deletions. Studies have shown that *pfhrp3* deletions are more prevalent than *pfhrp2* deletions in Colombia, which also has been found to be the case in Peru (Gamboa et al., 2010) and Honduras (Abdallah et al., 2015; Amoah et al., 2016). Remarkably, this is not the case in Surinam where *pfhrp2*-negative parasites were detected in higher numbers than *pfhrp3*-negative isolates (Akinyi et al., 2013).

The presence of parasite carrying both *pfhrp2* – and *pfhrp3* – have been reported. In some countries in South America, particularly those in the Amazon, a percentage as high as 25.7% has been observed (Amoah et al., 2016; Gamboa et al., 2010). In this study, the prevalence of parasites with *pfhrp2* –/*pfhrp3* – was 38.1% (8/21) and this was only in the samples from Accra. Previously, in 2016, a prevalence of double *pfhrp2* –/*pfhrp3* – parasites obtained in Accra over the months of February to May was 4.3 %, similar to the 3% that was obtained in samples collected in April (Amoah et al., 2016). *Plasmodium falciparum* parasites that lack part or all the *pfhrp2* gene do not express the PfHRP2 protein and were therefore not detectable by the *pfhrp2* detecting RDT kits. It is likely

that HRP2-detecting RDTs may have limited reliability for detecting *P. falciparum* in some parts of the Ghana. Consequently, RDTs that target other parasite antigens (pLDH and aldolase) and quality microscopy should be used for malaria diagnosis (WHO, 2017a, 2018) in such settings.

The potential impact of *P. falciparum* parasites lacking pfhrp2 on malaria case management and on procurement decisions regarding the type of RDTs is significant, and as such it is imperative that the presence and prevalence of these parasites are properly identified along a larger population to obtain a substantive evidence for the costly change of an RDT kit (Kyabayinze et al., 2016). These may go a long way to help in the fight to eliminate malaria.

Despite the significance of the study, certain limitations were faced. Due to limited funding, time and resources other assays such as msp gene PCR analysis could not be performed for parasite identification.

## 5.2 CONCLUSIONS

An overall RDT positivity rate of 7.3% (27/371) was observed. *Plasmodium falciparum* parasites that lack pfhrp2 genes alone or in addition to pfhrp3 genes were identified at two sites, Accra and Tafo, but not Kumasi. In this study, 14 negative-branded PfHRP2 RDT isolates, consisting of 13 (92.9%) samples from Accra and 1 (7.1%) from Tafo, were negative for the pfhrp2 gene (pfhrp2-). Only two samples from Accra, both negative-branded PfHRP2 RDT, were lacking the pfhrp3 gene (pfhrp3-).

It is recommended that:

1. The study should be extended to other districts in Accra as well as other regions in the country.
2. Decisions regarding the choice of malaria diagnosing test kits could be reviewed to suit the genetically dynamic species of *P. falciparum* malaria parasites in the nation as a whole.
3. Aldolase and pLDH enzymes, that are utilized by the parasite for its metabolism and survival, could be assayed, and their genetic bases amplified and compared to the *pfhrp2* / *pfhrp3* genes. If significant results are obtained in this study. RDT kits for our sub region could be engineered with antibodies to target these proteins so that genetic deletions resulting in false negative diagnosis would be reduced or totally prevented.

## REFERENCES

- Abba, K., Kirkham, A. J., Olliaro, P. L., Deeks, J. J., Donegan, S., Garner, P., & Takwoingi, Y. (2014). Rapid diagnostic tests for diagnosing uncomplicated non-falciparum or Plasmodium vivax malaria in endemic countries. *Cochrane Database Syst Rev*(12), Cd011431. doi:10.1002/14651858.cd011431
- Abdallah, J. F., Okoth, S. A., Fontecha, G. A., Torres, R. E., Banegas, E. I., Matute, M. L., . . . Udhayakumar, V. (2015). Prevalence of pfhrp2 and pfhrp3 gene deletions in Puerto Lempira, Honduras. *Malar J*, 14, 19. doi:10.1186/s12936-014-0537-7
- Adeoye, G. O., & Nga, I. C. (2007). Comparison of Quantitative Buffy Coat technique (QBC) with Giemsa-stained Thick Film (GTF) for diagnosis of malaria. *Parasitol Int*, 56(4), 308-312. doi:10.1016/j.parint.2007.06.007
- Akinyi, S., Hayden, T., Gamboa, D., Torres, K., Bendezu, J., Abdallah, J. F., . . . Udhayakumar, V. (2013). Multiple genetic origins of histidine-rich protein 2 gene deletion in Plasmodium falciparum parasites from Peru. *Sci Rep*, 3, 2797. doi:10.1038/srep02797
- Amir, A., Cheong, F. W., De Silva, J. R., & Lau, Y. L. (2018). Diagnostic tools in childhood malaria. *Parasit Vectors*, 11(1), 53. doi:10.1186/s13071-018-2617-y
- Amoah, L. E., Abankwa, J., & Oppong, A. (2016). Plasmodium falciparum histidine rich protein-2 diversity and the implications for PfHRP 2: based malaria rapid diagnostic tests in Ghana. *Malar J*, 15, 101. doi:10.1186/s12936-016-1159-z
- Aonuma, H., Suzuki, M., Iseki, H., Perera, N., Nelson, B., Igarashi, I., . . . Fukumoto, S. (2008). Rapid identification of Plasmodium-carrying mosquitoes using loop-mediated isothermal amplification. *Biochem Biophys Res Commun*, 376(4), 671-676. doi:10.1016/j.bbrc.2008.09.061
- Autino, B., Noris, A., Russo, R., & Castelli, F. (2012). Epidemiology of malaria in endemic areas. *Mediterr J Hematol Infect Dis*, 4(1), e2012060. doi:10.4084/mjhid.2012.060
- Baiden, F., Webster, J., Tivura, M., Delimini, R., Berko, Y., Amenga-Etego, S., . . . Chandramohan, D. (2012). Accuracy of rapid tests for malaria and treatment outcomes for malaria and non-malaria cases among under-five children in rural Ghana. *PLoS One*, 7(4), e34073. doi:10.1371/journal.pone.0034073
- Baird, J. K., & Hoffman, S. L. (2004). Primaquine therapy for malaria. *Clin Infect Dis*, 39(9), 1336-1345. doi:10.1086/424663
- Baker, J., Ho, M. F., Pelecanos, A., Gatton, M., Chen, N., Abdullah, S., . . . Cheng, Q. (2010). Global sequence variation in the histidine-rich proteins 2 and 3 of Plasmodium falciparum: implications for the performance of malaria rapid diagnostic tests. *Malar J*, 9, 129. doi:10.1186/1475-2875-9-129
- Baker, J., McCarthy, J., Gatton, M., Kyle, D. E., Belizario, V., Luchavez, J., . . . Cheng, Q. (2005). Genetic diversity of Plasmodium falciparum histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests. *J Infect Dis*, 192(5), 870-877. doi:10.1086/432010
- Barillas-Mury, C., & Kumar, S. (2005). Plasmodium-mosquito interactions: a tale of dangerous liaisons. *Cell Microbiol*, 7(11), 1539-1545. doi:10.1111/j.1462-5822.2005.00615.x
- Beadle, C., Long, G. W., Weiss, W. R., McElroy, P. D., Maret, S. M., Oloo, A. J., & Hoffman, S. L. (1994). Diagnosis of malaria by detection of Plasmodium falciparum HRP-2 antigen with a rapid dipstick antigen-capture assay. *Lancet*, 343(8897), 564-568. doi:10.1016/s0140-6736(94)91520-2

- Bell, D. R., Wilson, D. W., & Martin, L. B. (2005). False-positive results of a Plasmodium falciparum histidine-rich protein 2-detecting malaria rapid diagnostic test due to high sensitivity in a community with fluctuating low parasite density. *Am J Trop Med Hyg*, 73(1), 199-203.
- Bendezu, J., Rosas, A., Grande, T., Rodriguez, H., Llanos-Cuentas, A., Escobedo, J., & Gamboa, D. (2010). Field evaluation of a rapid diagnostic test (Parascreen) for malaria diagnosis in the Peruvian Amazon. *Malar J*, 9, 154. doi:10.1186/1475-2875-9-154
- Berhane, A., Russom, M., Bahta, I., Hagos, F., Ghirmai, M., & Uqubay, S. J. M. J. (2017). Rapid diagnostic tests failing to detect Plasmodium falciparum infections in Eritrea: an investigation of reported false negative RDT results. *16*(1), 105. doi:10.1186/s12936-017-1752-9
- Berzosa, P., de Lucio, A., Romay-Barja, M., Herrador, Z., Gonzalez, V., Garcia, L., . . . Benito, A. (2018). Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea. *Malar J*, 17(1), 333. doi:10.1186/s12936-018-2481-4
- Bhandari, P. L., Raghuvver, C. V., Rajeev, A., & Bhandari, P. D. (2008). Comparative study of peripheral blood smear, quantitative buffy coat and modified centrifuged blood smear in malaria diagnosis. *Indian J Pathol Microbiol*, 51(1), 108-112.
- Bisoffi, Z., Sirima, S. B., Menten, J., Pattaro, C., Angheben, A., Gobbi, F., . . . Van den Ende, J. (2010). Accuracy of a rapid diagnostic test on the diagnosis of malaria infection and of malaria-attributable fever during low and high transmission season in Burkina Faso. *Malar J*, 9, 192. doi:10.1186/1475-2875-9-192
- Boutlis, C. S., Riley, E. M., Anstey, N. M., & de Souza, J. B. (2005). Glycosylphosphatidylinositols in malaria pathogenesis and immunity: potential for therapeutic inhibition and vaccination. *Curr Top Microbiol Immunol*, 297, 145-185.
- Britton, S., Cheng, Q., & McCarthy, J. S. (2016). Novel molecular diagnostic tools for malaria elimination: a review of options from the point of view of high-throughput and applicability in resource limited settings. *Malar J*, 15, 88. doi:10.1186/s12936-016-1158-0
- Brown, W. M., Yowell, C. A., Hoard, A., Vander Jagt, T. A., Hunsaker, L. A., Deck, L. M., . . . Vander Jagt, D. L. (2004). Comparative structural analysis and kinetic properties of lactate dehydrogenases from the four species of human malarial parasites. *Biochemistry*, 43(20), 6219-6229. doi:10.1021/bi049892w
- Bruce-Chwatt, L. (1988). History of malaria from prehistory to eradication. In W. Wernsdorfer & I. McGregor (Eds.), *Malaria: Principles and Practice of Malariology* (Vol. 1, pp. 1-59). Edinburgh: Churchill Livingstone.
- Cadigan, F. C., & Desowitz, R. S. (1969). Two cases of Plasmodium ovale malaria from central Thailand. *Trans R Soc Trop Med Hyg*, 63(5), 681-682. doi:10.1016/0035-9203(69)90194-1
- CDC. (2018, November 14, 2018). About Malaria: Biology-Mosquitoes. Retrieved from <https://www.cdc.gov/malaria/about/biology/#tabs-1-5>
- Chauhan, V., Negi, R. C., Verma, B., & Thakur, S. (2009). Transfusion transmitted malaria in a non-endemic area. *J Assoc Physicians India*, 57, 654-656.
- Chotivanich, K., Silamut, K., & Day, N. P. J. (2006). Laboratory diagnosis of malaria infection-a short review of methods. *Aust J Med Sci.*, 27, 11-15.
- Clendennen, T. E., 3rd, Long, G. W., & Baird, J. K. (1995). QBC and Giemsa-stained thick blood films: diagnostic performance of laboratory technologists. *Trans R Soc Trop Med Hyg*, 89(2), 183-184. doi:10.1016/0035-9203(95)90486-7

- Collins, W. E., & Jeffery, G. M. (2005). Plasmodium ovale: parasite and disease. *Clin Microbiol Rev*, 18(3), 570-581. doi:10.1128/cmr.18.3.570-581.2005
- Collins, W. E., & Jeffery, G. M. (2007). Plasmodium malariae: parasite and disease. *Clin Microbiol Rev*, 20(4), 579-592. doi:10.1128/cmr.00027-07
- Cox-Singh, J., Davis, T. M., Lee, K. S., Shamsul, S. S., Matusop, A., Ratnam, S., . . . Singh, B. (2008). Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis*, 46(2), 165-171. doi:10.1086/524888
- Day, N. P., Hien, T. T., Schollaardt, T., Loc, P. P., Chuong, L. V., Chau, T. T., . . . Ho, M. (1999). The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. *J Infect Dis*, 180(4), 1288-1297. doi:10.1086/315016
- Desakorn, V., Dondorp, A. M., Silamut, K., Pongtavornpinyo, W., Sahassananda, D., Chotivanich, K., . . . White, N. J. (2005). Stage-dependent production and release of histidine-rich protein 2 by Plasmodium falciparum. *Trans R Soc Trop Med Hyg*, 99(7), 517-524. doi:10.1016/j.trstmh.2004.11.014
- Dhiman, S., Yadav, K., Goswami, D., Das, N., Baruah, I., & Singh, L. (2012). Epidemiology and Risk Analysis of Malaria among Pregnant Women. *Iran J Public Health*, 41(1), 1-8.
- Dobeli, H., Trzeciak, A., Gillessen, D., Matile, H., Srivastava, I. K., Perrin, L. H., . . . Certa, U. (1990). Expression, purification, biochemical characterization and inhibition of recombinant Plasmodium falciparum aldolase. *Mol Biochem Parasitol*, 41(2), 259-268.
- Dondorp, A. M., Desakorn, V., Pongtavornpinyo, W., Sahassananda, D., Silamut, K., Chotivanich, K., . . . Day, N. P. (2005). Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. *PLoS Med*, 2(8), e204. doi:10.1371/journal.pmed.0020204
- Dzakah, E. E., Kang, K., Ni, C., Tang, S., Wang, J., & Wang, J. (2014). Comparative performance of aldolase and lactate dehydrogenase rapid diagnostic tests in Plasmodium vivax detection. *Malar J*, 13, 272. doi:10.1186/1475-2875-13-272
- Endeshaw, T., Gebre, T., Ngondi, J., Graves, P. M., Shargie, E. B., Ejigsemahu, Y., . . . Richards, F. O. (2008). Evaluation of light microscopy and rapid diagnostic test for the detection of malaria under operational field conditions: a household survey in Ethiopia. *Malar J*, 7, 118. doi:10.1186/1475-2875-7-118
- Fançony, C., Sebastião, Y. V., Pires, J. E., Gamboa, D., & Nery, S. V. (2013). Performance of microscopy and RDTs in the context of a malaria prevalence survey in Angola: a comparison using PCR as the gold standard. *Malar J*, 12, 284. doi:10.1186/1475-2875-12-284
- Faye, F. B., Konate, L., Rogier, C., & Trape, J. F. (1998). Plasmodium ovale in a highly malaria endemic area of Senegal. *Trans R Soc Trop Med Hyg*, 92(5), 522-525. doi:10.1016/s0035-9203(98)90900-2
- Forney, J. R., Wongsrichanalai, C., Magill, A. J., Craig, L. G., Sirichaisinthop, J., Bautista, C. T., . . . Gasser, R. A., Jr. (2003). Devices for rapid diagnosis of Malaria: evaluation of prototype assays that detect Plasmodium falciparum histidine-rich protein 2 and a Plasmodium vivax-specific antigen. *J Clin Microbiol*, 41(6), 2358-2366. doi:10.1128/jcm.41.6.2358-2366.2003
- Freimanis, G., Sedegah, M., Owusu-Ofori, S., Kumar, S., & Allain, J. P. (2013). Investigating the prevalence of transfusion transmission of Plasmodium within a hyperendemic blood donation system. *Transfusion*, 53(7), 1429-1441. doi:10.1111/j.1537-2995.2012.03943.x

- Fried, M., & Duffy, P. E. (2017). Malaria during Pregnancy. *Cold Spring Harb Perspect Med*, 7(6). doi:10.1101/cshperspect.a025551
- Fuehrer, H. P., Starzengruber, P., Swoboda, P., Khan, W. A., Matt, J., Ley, B., . . . Noedl, H. (2010). Indigenous Plasmodium ovale malaria in Bangladesh. *Am J Trop Med Hyg*, 83(1), 75-78. doi:10.4269/ajtmh.2010.09-0796
- Gamboa, D., Ho, M. F., Bendezu, J., Torres, K., Chiodini, P. L., Barnwell, J. W., . . . Cheng, Q. (2010). A large proportion of P. falciparum isolates in the Amazon region of Peru lack pfhrp2 and pfhrp3: implications for malaria rapid diagnostic tests. *PLoS One*, 5(1), e8091. doi:10.1371/journal.pone.0008091
- Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., . . . Barrell, B. (2002). Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature*, 419(6906), 498-511. doi:10.1038/nature01097
- Gatti, S., Gramegna, M., Bisoffi, Z., Raglio, A., Gulletta, M., Klersy, C., . . . Scaglia, M. (2007). A comparison of three diagnostic techniques for malaria: a rapid diagnostic test (NOW Malaria), PCR and microscopy. *Ann Trop Med Parasitol*, 101(3), 195-204. doi:10.1179/136485907x156997
- Gleason, N. N., Fisher, G. U., Blumhardt, R., Roth, A. E., & Gaffney, G. W. (1970). Plasmodium ovale malaria acquired in Viet-Nam. *Bull World Health Organ*, 42(3), 399-403.
- Grobusch, M. P., Hanscheid, T., Kramer, B., Neukammer, J., May, J., Seybold, J., . . . Suttorp, N. (2003). Sensitivity of hemozoin detection by automated flow cytometry in non- and semi-immune malaria patients. *Cytometry B Clin Cytom*, 55(1), 46-51. doi:10.1002/cyto.b.10039
- Gupta, H., Matambisso, G., Galatas, B., Cisteró, P., Nhamussua, L., Simone, W., . . . Mayor, A. (2017). Molecular surveillance of pfhrp2 and pfhrp3 deletions in Plasmodium falciparum isolates from Mozambique. *Malar J*, 16. doi:10.1186/s12936-017-2061-z
- Hanscheid, T., & Grobusch, M. P. (2002). How useful is PCR in the diagnosis of malaria? *Trends Parasitol.*, 18, 395–398.
- Haque, U., Ahmed, S. M., Hossain, S., Huda, M., Hossain, A., Alam, M. S., . . . Haque, R. (2009). Malaria prevalence in endemic districts of Bangladesh. *PLoS One*, 4(8), e6737. doi:10.1371/journal.pone.0006737
- Harani, M. S., Beg, M. A., Khaleeq, L., Adil, S. N., Kakepoto, G. N., & Khurshid, M. (2006). Role of ICT malaria immunochromatographic test for rapid diagnosis of malaria. *J Pak Med Assoc*, 56(4), 167-171.
- Holland, C. A., & Kiechle, F. L. (2005). Point-of-care molecular diagnostic systems--past, present and future. *Curr Opin Microbiol*, 8(5), 504-509. doi:10.1016/j.mib.2005.08.001
- Houze, S., Hubert, V., Cohen, D. P., Rivetz, B., & Le Bras, J. (2011). Evaluation of the Clearview(R) Malaria pLDH Malaria Rapid Diagnostic Test in a non-endemic setting. *Malar J*, 10, 284. doi:10.1186/1475-2875-10-284
- Howard, R. J., Uni, S., Aikawa, M., Aley, S. B., Leech, J. H., Lew, A. M., . . . Taylor, D. W. (1986). Secretion of a malarial histidine-rich protein (Pf HRP II) from Plasmodium falciparum-infected erythrocytes. *J Cell Biol*, 103(4), 1269-1277.
- Humar, A., Ohrt, C., Harrington, M. A., Pillai, D., & Kain, K. C. (1997). Parasight F test compared with the polymerase chain reaction and microscopy for the diagnosis of Plasmodium falciparum malaria in travelers. *Am J Trop Med Hyg*, 56(1), 44-48. doi:10.4269/ajtmh.1997.56.44
- Imwong, M., Pukrittayakamee, S., Pongtavornpinyo, W., Nakeesathit, S., Nair, S., Newton, P., . . . White, N. J. (2008). Gene amplification of the multidrug

- resistance 1 gene of *Plasmodium vivax* isolates from Thailand, Laos, and Myanmar. *Antimicrob Agents Chemother*, *52*(7), 2657-2659. doi:10.1128/aac.01459-07
- Iqbal, J., Sher, A., & Rab, A. (2000). Plasmodium falciparum histidine-rich protein 2-based immunocapture diagnostic assay for malaria: cross-reactivity with rheumatoid factors. *J Clin Microbiol*, *38*(3), 1184-1186.
- Izumiyama, S., Omura, M., Takasaki, T., Ohmae, H., & Asahi, H. (2009). Plasmodium falciparum: development and validation of a measure of intraerythrocytic growth using SYBR Green I in a flow cytometer. *Exp Parasitol*, *121*(2), 144-150. doi:10.1016/j.exppara.2008.10.008
- Keating, J., Miller, J. M., Bennett, A., Moonga, H. B., & Eisele, T. P. (2009). Plasmodium falciparum parasite infection prevalence from a household survey in Zambia using microscopy and a rapid diagnostic test: implications for monitoring and evaluation. *Acta Trop*, *112*(3), 277-282. doi:10.1016/j.actatropica.2009.08.011
- Kifude, C. M., Rajasekariah, H. G., Sullivan, D. J., Jr., Stewart, V. A., Angov, E., Martin, S. K., . . . Waitumbi, J. N. (2008). Enzyme-linked immunosorbent assay for detection of Plasmodium falciparum histidine-rich protein 2 in blood, plasma, and serum. *Clin Vaccine Immunol*, *15*(6), 1012-1018. doi:10.1128/cvi.00385-07
- Kiszewski, A., Mellinger, A., Spielman, A., Malaney, P., Sachs, S. E., & Sachs, J. (2004). A global index representing the stability of malaria transmission. *Am J Trop Med Hyg*, *70*(5), 486-498.
- Knapp, B., Hundt, E., & Kupper, H. A. (1990). Plasmodium falciparum aldolase: gene structure and localization. *Mol Biochem Parasitol*, *40*(1), 1-12.
- Koita, O. A., Doumbo, O. K., Ouattara, A., Tall, L. K., Konaré, A., Diakit , M., . . . Krogstad, D. J. (2012). False-Negative Rapid Diagnostic Tests for Malaria and Deletion of the Histidine-Rich Repeat Region of the hrp2 Gene. *Am J Trop Med Hyg*, *86*(2), 194-198. doi:10.4269/ajtmh.2012.10-0665
- Kumar, N., Singh, J. P., Pande, V., Mishra, N., Srivastava, B., Kapoor, R., . . . Anvikar, A. R. (2012). Genetic variation in histidine rich proteins among Indian Plasmodium falciparum population: possible cause of variable sensitivity of malaria rapid diagnostic tests. *Malar J*, *11*, 298. doi:10.1186/1475-2875-11-298
- Kurtzhals, J. A., Adabayeri, V., Goka, B. Q., Akanmori, B. D., Oliver-Commey, J. O., Nkrumah, F. K., . . . Hviid, L. (1998). Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. *Lancet*, *351*(9118), 1768-1772. doi:10.1016/s0140-6736(97)09439-7
- Kyabayinze, D. J., Zongo, I., Cunningham, J., Gatton, M., Angutoko, P., Ategeka, J., . . . Bell, D. (2016). HRP2 and pLDH-Based Rapid Diagnostic Tests, Expert Microscopy, and PCR for Detection of Malaria Infection during Pregnancy and at Delivery in Areas of Varied Transmission: A Prospective Cohort Study in Burkina Faso and Uganda. *PLoS One*, *11*(7), e0156954. doi:10.1371/journal.pone.0156954
- Lee, N., Baker, J., Andrews, K. T., Gatton, M. L., Bell, D., Cheng, Q., & McCarthy, J. (2006). Effect of sequence variation in Plasmodium falciparum histidine-rich protein 2 on binding of specific monoclonal antibodies: Implications for rapid diagnostic tests for malaria. *J Clin Microbiol*, *44*(8), 2773-2778. doi:10.1128/jcm.02557-05
- Lee, N., Gatton, M. L., Pelecanos, A., Bubb, M., Gonzalez, I., Bell, D., . . . McCarthy, J. S. (2012). Identification of optimal epitopes for Plasmodium falciparum rapid diagnostic tests that target histidine-rich proteins 2 and 3. *J Clin Microbiol*, *50*(4), 1397-1405. doi:10.1128/jcm.06533-11

- Lysenko, A. J., & Beljaev, A. E. (1969). An analysis of the geographical distribution of *Plasmodium ovale*. *Bull World Health Organ*, 40(3), 383-394.
- Makler, M. T., Piper, R. C., & Milhous, W. K. (1998). Lactate dehydrogenase and the diagnosis of malaria. *Parasitol Today*, 14(9), 376-377.
- Maltha, J., Gillet, P., & Jacobs, J. (2013a). Malaria rapid diagnostic tests in endemic settings. *Clin Microbiol Infect*, 19(5), 399-407. doi:10.1111/1469-0691.12151
- Maltha, J., Gillet, P., & Jacobs, J. (2013b). Malaria rapid diagnostic tests in travel medicine. *Clin Microbiol Infect*, 19(5), 408-415. doi:10.1111/1469-0691.12152
- Maltha, J., Guiraud, I., Lompo, P., Kabore, B., Gillet, P., Van Geet, C., . . . Jacobs, J. (2014). Accuracy of PfHRP2 versus Pf-pLDH antigen detection by malaria rapid diagnostic tests in hospitalized children in a seasonal hyperendemic malaria transmission area in Burkina Faso. *Malar J*, 13, 20. doi:10.1186/1475-2875-13-20
- Mens, P., Spieker, N., Omar, S., Heijnen, M., Schallig, H., & Kager, P. A. (2007). Is molecular biology the best alternative for diagnosis of malaria to microscopy? A comparison between microscopy, antigen detection and molecular tests in rural Kenya and urban Tanzania. *Trop Med Int Health*, 12(2), 238-244. doi:10.1111/j.1365-3156.2006.01779.x
- Moody, A. (2002). Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev*, 15(1), 66-78.
- Morassin, B., Fabre, R., Berry, A., & Magnaval, J. F. (2002). One year's experience with the polymerase chain reaction as a routine method for the diagnosis of imported malaria. *Am J Trop Med Hyg*, 66(5), 503-508. doi:10.4269/ajtmh.2002.66.503
- Mouatcho, J. C., & Goldring, J. P. (2013). Malaria rapid diagnostic tests: challenges and prospects. (1473-5644 (Electronic)).
- Nkrumah, B., Acquah, S. E., Ibrahim, L., May, J., Brattig, N., Tannich, E., . . . Huenger, F. (2011). Comparative evaluation of two rapid field tests for malaria diagnosis: Partec Rapid Malaria Test(R) and Binax Now(R) Malaria Rapid Diagnostic Test. *BMC Infect Dis*, 11, 143. doi:10.1186/1471-2334-11-143
- Okell, L. C., Ghani, A. C., Lyons, E., & Drakeley, C. J. (2009). Submicroscopic infection in *Plasmodium falciparum*-endemic populations: a systematic review and meta-analysis. *J Infect Dis*, 200(10), 1509-1517. doi:10.1086/644781
- Palacios, G., Quan, P. L., Jabado, O. J., Conlan, S., Hirschberg, D. L., Liu, Y., . . . Lipkin, W. I. (2007). Panmicrobial oligonucleotide array for diagnosis of infectious diseases. *Emerg Infect Dis*, 13(1), 73-81. doi:10.3201/eid1301.060837
- Piera, K. A., Aziz, A., William, T., Bell, D., Gonzalez, I. J., Barber, B. E., . . . Grigg, M. J. (2017). Detection of *Plasmodium knowlesi*, *Plasmodium falciparum* and *Plasmodium vivax* using loop-mediated isothermal amplification (LAMP) in a co-endemic area in Malaysia. *Malar J*, 16(1), 29. doi:10.1186/s12936-016-1676-9
- Piper, R., Lebras, J., Wentworth, L., Hunt-Cooke, A., Houze, S., Chiodini, P., & Makler, M. (1999). Immunocapture diagnostic assays for malaria using *Plasmodium* lactate dehydrogenase (pLDH). *Am J Trop Med Hyg*, 60(1), 109-118. doi:10.4269/ajtmh.1999.60.109
- Poon, L. L., Wong, B. W., Ma, E. H., Chan, K. H., Chow, L. M., Abeyewickreme, W., . . . Peiris, J. S. (2006). Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. *Clin Chem*, 52(2), 303-306. doi:10.1373/clinchem.2005.057901
- Poser, C., & Bruyn, G. (1999). *An Illustrated History of Malaria*. New York: Parthenon.

- Rakotonirina, H., Barnadas, C., Rahejafy, R., Andrianantenaina, H., Ratsimbaoa, A., Randrianasolo, L., . . . Menard, D. (2008). Accuracy and reliability of malaria diagnostic techniques for guiding febrile outpatient treatment in malaria-endemic countries. *Am J Trop Med Hyg*, *78*(2), 217-221.
- Reyburn, H., Mbakilwa, H., Mwangi, R., Mwerinde, O., Olomi, R., Drakeley, C., & Whitty, C. J. (2007). Rapid diagnostic tests compared with malaria microscopy for guiding outpatient treatment of febrile illness in Tanzania: randomised trial. *Bmj*, *334*(7590), 403. doi:10.1136/bmj.39073.496829.AE
- Rock, E. P., Marsh, K., Saul, A. J., Wellems, T. E., Taylor, D. W., Maloy, W. L., & Howard, R. J. (1987). Comparative analysis of the Plasmodium falciparum histidine-rich proteins HRP-I, HRP-II and HRP-III in malaria parasites of diverse origin. *Parasitology*, *95* ( Pt 2), 209-227.
- Schindler, T., Robaina, T., Sax, J., Bieri, J. R., Mpina, M., Gondwe, L., . . . Daubenberg, C. (2019). Molecular monitoring of the diversity of human pathogenic malaria species in blood donations on Bioko Island, Equatorial Guinea. *Malar J*, *18*(1), 9. doi:10.1186/s12936-019-2639-8
- Schofield, L., & Hackett, F. (1993). Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. *J Exp Med*, *177*(1), 145-153. doi:10.1084/jem.177.1.145
- Scholl, P. F., Kongkasuriyachai, D., Demirev, P. A., Feldman, A. B., Lin, J. S., Sullivan, D. J., Jr., & Kumar, N. (2004). Rapid detection of malaria infection in vivo by laser desorption mass spectrometry. *Am J Trop Med Hyg*, *71*(5), 546-551.
- She, R. C., Rawlins, M. L., Mohl, R., Perkins, S. L., Hill, H. R., & Litwin, C. M. (2007). Comparison of immunofluorescence antibody testing and two enzyme immunoassays in the serologic diagnosis of malaria. *J Travel Med*, *14*(2), 105-111. doi:10.1111/j.1708-8305.2006.00087.x
- Singh, B., Bobogare, A., Cox-Singh, J., Snounou, G., Abdullah, M. S., & Rahman, H. A. (1999). A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *Am J Trop Med Hyg*, *60*(4), 687-692.
- Stephens, J. W. W. (1922). A new malaria parasite of man. *Ann Trop Med Parasito*, *16*, 383-388.
- Sulzer, A. J., Wilson, M., & Hall, E. C. (1969). Indirect fluorescent-antibody tests for parasitic diseases. V. An evaluation of a thick-smear antigen in the IFA test for malaria antibodies. *Am J Trop Med Hyg*, *18*(2), 199-205.
- Tangpukdee, N., Duangdee, C., Wilairatana, P., & Krudsood, S. (2009). Malaria diagnosis: a brief review. *Korean J Parasitol*, *47*(2), 93-102. doi:10.3347/kjp.2009.47.2.93
- Tham, J. M., Lee, S. H., Tan, T. M., Ting, R. C., & Kara, U. A. (1999). Detection and species determination of malaria parasites by PCR: comparison with microscopy and with ParaSight-F and ICT malaria Pf tests in a clinical environment. *J Clin Microbiol*, *37*(5), 1269-1273.
- Tritten, L., Matile, H., Brun, R., & Wittlin, S. (2009). A new double-antibody sandwich ELISA targeting Plasmodium falciparum aldolase to evaluate anti-malarial drug sensitivity. *Malar J*, *8*, 226. doi:10.1186/1475-2875-8-226
- Valecha, N., Bhatia, S., Mehta, S., Biswas, S., & Dash, A. P. (2007). Congenital malaria with atypical presentation: a case report from low transmission area in India. *Malar J*, *6*, 43. doi:10.1186/1475-2875-6-43
- Weir, W. (1997). Two cases of malaria: four messages. *Euro Surveill*, *1*(12). Retrieved from <https://doi.org/10.2807/esw.01.12.01077-en>

- Weiss, D. J., Lucas, T. C. D., Nguyen, M., Nandi, A. K., Bisanzio, D., Battle, K. E., . . . Gething, P. W. (2019). Mapping the global prevalence, incidence, and mortality of *Plasmodium falciparum*, 2000-17: a spatial and temporal modelling study. *Lancet*. doi:10.1016/s0140-6736(19)31097-9
- Wellems, T. E., & Howard, R. J. (1986). Homologous genes encode two distinct histidine-rich proteins in a cloned isolate of *Plasmodium falciparum*. *Proc Natl Acad Sci U S A*, 83(16), 6065-6069.
- White, N. J. (2008). *Plasmodium knowlesi*: the fifth human malaria parasite. *Clin Infect Dis*, 46(2), 172-173. doi:10.1086/524889
- WHO. (2011). *Report on the regional workshop on strengthening quality management systems for parasitological diagnosis of malaria*. Retrieved from [http://applications.emro.who.int/docs/IC\\_Meet\\_Rep\\_2012\\_EN\\_14512.pdf](http://applications.emro.who.int/docs/IC_Meet_Rep_2012_EN_14512.pdf)
- WHO. (2012). Management of severe malaria.
- WHO. (2014). Severe malaria. *Trop Med Int Health*, 19 Suppl 1(Special Issue), 7-131. doi:10.1111/tmi.12313\_2
- WHO. (2015a). Treatment of uncomplicated malaria caused by *P. vivax*, *P. ovale*, *P. malariae* or *P. knowlesi*. In *Guidelines for the Treatment of Malaria*. Geneva: World Health Organization.
- WHO. (2015b). *WHO-FIND malaria RDT evaluation programme: product testing round 5*. Retrieved from <http://www.who.int/malaria/mpac/mpac-sept2014-round5-product-testing-presentation.pdf>
- WHO. (2016a). *Methods Manual for Laboratory Quality Control Testing of Malaria RDTs (version 8)*. Retrieved from Geneva:
- WHO. (2016b). *World Malaria Report, 2016*. Retrieved from <http://www.who.int/malaria/publications/world-malariareport-2016/report/en/>
- WHO. (2017a). *False-negative RDT results and implications of new reports of P. falciparum histidine-rich protein 2/3 gene deletions*. Retrieved from [apps.who.int/iris/bitstream/10665/258972/1/WHO-HTM-GMP-2017.18-eng.pdf](http://apps.who.int/iris/bitstream/10665/258972/1/WHO-HTM-GMP-2017.18-eng.pdf)
- WHO. (2017b). *Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs: round 7 (2015-2016)*. Retrieved from <http://www.who.int/malaria/publications/atoz/978924151268/en/>
- WHO. (2017c). *World Malaria Report 2017*. Retrieved from <http://afro.who.int/publications/world-malaria-report-2017>
- WHO. (2018). *World Malaria Report 2018*. Retrieved from Geneva: <https://www.who.int/malaria/publications/world-malaria-report-2018/en/>
- WHO. (2019). Malaria: key facts. In (27th March, 2019 ed.). Geneva: World Health Organisation.
- Wongsrichanalai, C., Barcus, M. J., Muth, S., Sutamihardja, A., & Wernsdorfer, W. H. (2007). A Review of Malaria Diagnostic Tools: Microscopy and Rapid Diagnostic Test (RDT). In Breman JG, Alilio MS, & W. NJ (Eds.), *Defining and Defeating the Intolerable Burden of Malaria III: Progress and Perspectives* (Vol. Supplement to Volume 77(6)): American Society of Tropical Medicine and Hygiene.

## APPENDIXES

## Appendix I

Table 1: Details of oligonucleotides primers for PCR amplifications.

Purpose	Primer	Sequence (5'-3')
<i>Plasmodium</i> sp. <sup>1</sup>	rPLU5	CTTGTTGTTGCCTTAAACTTC
	rPLU6	TTAAAATTGTTGCAGTTAAAACG
<i>P. falciparum</i> <sup>1</sup>	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT
	rFAL2	ACACAATGAACTCAATCATGACTACCCGTC
<i>msp-2</i> <sup>1</sup>	msp2-C1	ATGAAGGTAATTA AACATTGTCTATTATA
	msp2-C2	CTTTGTTACCATCGGTACATTCTT
	FC27a	AATACTAAGAGTGTAGGTGCA(AG)ATGCTCCA
	FC27b	TTTTATTTGGTGCATTGCCAGA ACTTGAAC
	3D7a	AGAAGTATGGCAGAAAGTAA(GT)CCT(CT)CTACT
	3D7b	GATTGTAATTCGGGGGATT CAGTTTGTTCG
	<i>Pfhrp</i> <sup>2</sup>	Pfhrp2-F1
	Pfhrp2-R1	AATAAATTTAATGGCGTAGGCA
	Pfhrp2-F2	ATTATTACACGAAACTCAAGCAC
<i>Pfhrp3</i> <sup>2</sup>	Pfhrp3-F1	AATGCAAAAGGACTTAATTC
	Pfhrp3-R1	TGGTGTAAGTGATGCGTAGT
	Pfhrp3-F2	AAATAAGAGATTATTACACGAAAG

<sup>1</sup>Singh et al., 1999; <sup>2</sup>Baker et al. 2005.

## Appendix IIA

### CONSENT FORM

#### **Title: GENETIC VARIATION OF PFHRP2 IN *PLASMODIUM FALCIPARUM* ISOLATES AND THE PERFORMANCE OF HRP2 BASED MALARIA RAPID DIAGNOSTIC TESTS**

**Name and Affiliation of Researcher:** This Study Is Being Conducted by Edith Bortier of the Department of Medical Laboratory Sciences, School of Biomedical and Allied Health Sciences (SBAHS), College of Health Sciences, University of Ghana.

#### **General Information About Research**

Malaria still remains the world's most prevalent vector-borne disease due to its relatively high and alarming morbidity and mortality rates. An accurate diagnosis is essential for the rapid and appropriate treatment of malaria and malaria rapid diagnostic tests (RDTs) play a key role in this regard. The *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) gene based RDT is the most widely used RDT for malaria diagnosis in Ghana. Deletion of *pfhrp2* in field isolates of *P. falciparum* can therefore result in false negative test results when PfHRP2 RDTs are used for malaria diagnosis. Some PfHRP2-detecting RDTs are known to cross react with PfHRP3. Identifying the prevalence and distribution of *P. falciparum* parasites with deleted *pfhrp2* and/or *pfhrp3* is important for malaria control.

You are being invited to participate in a research project that is being conducted at 4 sites in Ghana. The aim of this study is to determine the prevalence of *pfhrp2* and/or *pfhrp3* genes and their impact on *pfhrp*-based RDTs in natural *P. falciparum* parasite populations.

You will be contacted by the laboratory staff when come to do a malaria test. Your will be included in the study if you agree to be part of the study and its procedures. After you have been pricked for the malaria test, a drop of your blood will be collected on to filter paper. This will done be done under sterile conditions. A sterile prick at finger will be done to collect blood.

### **Possible Risks and Discomforts**

There is minimal risk to you if you agree to participate in the study. Finger-prick blood collection may cause some discomfort, pain, swelling and rarely infection at the site where the prick is done. Competent and experienced health personnel will ensure that this potential discomfort is minimized.

### **Possible Benefits**

Information on genetic variation will help to analyse and critically ascertain the use of the RDT kits in order to eliminate improper diagnosis that leads to prolong treatment of malaria infections. If there is the establishment of the fact that certain deletions do not allow the RDT kit to pick up some strains of malaria parasite leading to false negative results, policy makers would be able to make policy changes to use other alternatives or improve the RDT detection limit. This will further aid in the reduction in malaria morbidity and mortality.

### **Confidentiality**

If you decide to participate in this study, the health-provider will collect medical and personal information about you as part of the study. People who work for or with the sponsor, and others like the independent ethics committee or the institutional review board (IRB) for the study, will have access to this information at the site. They will check to see if the study is going on well and to ensure your rights. All staff that review your information at the site will keep it confidential. All records about you will be kept in locked cabinets.

### **Voluntary Participation and Right to Leave the Research**

Participation is entirely voluntary and refusal will not result in penalty or loss of benefit. You can withdraw on your own from the study at any point in time during the duration of the study. You will not be required to give any reason for this action. However, information collected from you will be used as part of the study's analysis. You will not be denied access to health if you withdraw from the study

### **Contacts for Additional Information**

If you have any questions about this study, if there are things that you do not understand, or if your child has a study-related injury or hospitalized, please contact: Dr. Charles

Brown at the Department of Medical Laboratory Sciences, School of Biomedical and Allied Health Sciences (SBAHS), College of Health Sciences, University of Ghana. 030 2501178/9

**Your Rights as a Participant**

This research has been reviewed and approved by the Ethics and Protocol Review Committee (College of Health Sciences, University of Ghana). If you have any questions about your rights as a research participant you can contact my supervisor:

Dr Charles Brown, Department of Medical Laboratory Sciences, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana, Korle bu Campus. Tel: 0200242072

**VOLUNTEER AGREEMENT**

The above document describing the benefits, risks and procedures for the research title (Genetic variation of pfhrp2 in *Plasmodium falciparum* isolates and the performance of hrp2 based malaria rapid diagnostic tests) has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

\_\_\_\_\_  
**Name and signature or mark of volunteer**

\_\_\_\_\_  
**Date**

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

\_\_\_\_\_  
**Name and signature of witness**

\_\_\_\_\_  
**Date**

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

\_\_\_\_\_  
**Name Signature of Person Who Obtained  
Consent**

\_\_\_\_\_  
**Date**

## **Appendix IIB**

### **CHILD ASSENT FORM**

#### **Introduction**

My name is *Edith Bortier* and I am from the *Department of Medical Laboratory Sciences* at *School of Biomedical and Allied Health Sciences, University of Ghana*. I am conducting a research study entitled “*Genetic variation of pfhrp2 in Plasmodium falciparum isolates and the performance of hrp2 based malaria rapid diagnostic tests*”. I am asking you to take part in this research study because I am trying to *learn more about false negative test results when PfHRP2 RDTs are used for malaria diagnosis*. This will take *less than 10 minutes of your times*.

#### **General Information**

If you agree to be in this study, you will be asked *to provide a drop of your blood which will be collected on to filter paper*.

#### **Possible Benefits**

Your participation in this study will result in *information on genetic variation will help to analyse and critically ascertain the use of the RDT kits in order to eliminate improper diagnosis that leads to prolong treatment of malaria infections*.

#### **Possible Risks and Discomforts**

However, the risks associated *is minimal risk to you if you agree to participate in the study*.

#### **Voluntary Participation and Right to Leave the Research**

You can stop participating at any time if you feel uncomfortable. No one will be angry with you if you do not want to participate.

#### **Confidentiality**

Your information will be kept confidential. No one will be able to know how you responded to the questions and your information will be anonymous.

**Contacts for Additional Information**

You may ask me any questions about this study. You can call me at any time on 0207575933/0244693968 or talk to me the next time you see me.

Please talk about this study with your parents before you decide whether or not to participate. I will also ask permission from your parents before you are enrolled into the study. Even if your parents say “yes” you can still decide not to participate.

**Your rights as a Participant**

This research has been reviewed and approved by the Ethics and Protocol Review Committee (College of Health Sciences, University of Ghana). If you have any questions about your rights as a research participant you can contact the EPRC Office between the hours of 8 am-5 pm through the landline 0306665103/4 or email addresses: [admin.chs@chs.edu.gh](mailto:admin.chs@chs.edu.gh)

**VOLUNTARY AGREEMENT**

By making a mark or thumb printing below, it means that you understand and know the issues concerning this research study. If you do not want to participate in this study, please do not sign this assent form. You and your parents will be given a copy of this form after you have signed it.

This assent form which describes the benefits, risks and procedures for the research titled *Genetic variation of pfhrp2 in Plasmodium falciparum isolates and the performance of hrp2 based malaria rapid diagnostic tests* has been read and or explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate.

---

**Child's Name**

---

**Researcher's Name**

---

**Child's Mark/Thumbprint**

---

**Researcher's Signature**

---

**Date**

---

**Date**