UNIVERSITY FOR DEVELOPMENT STUDIES

PLASMODIUM FALCIPARUM HISTIDINE-RICH PROTEIN GENE MUTATIONS IN NORTHERN GHANA: IMPLICATIONS FOR THE USE OF HRP2-BASED MALARIA DIAGNOSIS

GIDEON ADU-BONSU

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BY

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(BSc. BIOTECHNOLOGY AND MOLECULAR BIOLOGY)

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THESIS SUBMITTED TO THE DEPARTMENT OF BIOTECHNOLOGY, FACULTY OF BIOSCIENCES, UNIVERSITY FOR DEVELOPMENT STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN BIOTECHNOLOGY

OCTOBER, 2022

DECLARATION

Student

I hereby declare that this thesis is the result of my original work and that no part of it has been presented for another degree in this University or elsewhere:

Candidate:

Signature: ……..…… Date: ……...……..

Name: Gideon Adu-Bonsu

Supervisors

We hereby declare that the preparation and presentation of the thesis was supervised following the guidelines on supervision of thesis laid down by the University for Development Studies.

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ABSTRACT

Human malaria is caused by five *Plasmodium* species transmitted by the female *Anopheles* mosquitoes. PCR assays, microscopy and RDT (most convenient), are malaria diagnostic approaches. RDT works on the principle of MAbs detecting antigens expressed by specific markers of *Plasmodium* spp. like the *Pfhrp2/3* genes. This study sought to determine the rate of *Pfhrp*2/3 gene mutations in northern Ghana and the implications of these mutations for the use of *Pf*HRP2-RDTs for malaria diagnosis in the region. Blood samples from 267 malaria patients diagnosed by microscopy were tested for malaria infection by *Pf*HRP2/3-based RDT and PCR using whole blood and extracted DNA, respectively. Nested PCR was also used to investigate the presence/absence of the *Pfhrp*2/3 genes and genetic variation of nucleotide and in-silico translated amino acids done using GenTLE, MEGAX and popART software. In all, 160 (59.9%) of the total samples tested positive to *P. falciparum*-specific PCR with 158/267 (59.2 %) positive by *Pf*HRP2-based RDT. A false RDT negative rate of 21.9% (35/160) was, however observed which can be attributed to several factors, including mutation or deletion of the target antigenic marker. PCR assay revealed that 19 (11.9 %) and 22 (13.8 %) samples lacked exon1−2 of *Pfhrp*2 and 3 genes, respectively with 17 (10.6%) samples lacking both genes. Twenty (20) of the false RDT negative samples lacked either or both exon1−2 of *Pfhrp*2 and 3 genes. Genetic variation studies revealed higher variability indices for both nucleotide sequences and amino acid residues of the sequenced exon1 of *Pfhrp*2 and 3 genes. Haplotype diversity was 0.925 (π=0.05273) and 1.000 (π=0.13517) for intron sequences of *Pfhrp*2 and 3, respectively. Isolates of this study formed a monophyletic clade but a paraphyletic clade with other isolates. This study revealed mutations to the *Pfhrp2/3* genes and raise concerns about the reliability of *Pf*HRP2/3-RDT for malaria diagnosis.

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DEDICATION

I dedicate this thesis to my parents, siblings, supervisors and all loved ones.

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LIST OF ACRONYMS, ABBREVIATIONS AND SYMBOLS

ACT	–	Artemisinin-based Combination Therapy
BLAST	–	Basic Local Alignment Search Tool
DNA	–	Deoxyribonucleic Acid
DnaSP	–	DNA Sequence Polymorphism
dNTPs	–	Deoxyribonucleotide Triphosphates
EBI	–	European Bioinformatics Institute
EDTA	–	Ethylenediamine-Tetra-Acetic Acid
EMBL	–	European Molecular Biology Laboratory-
GHS	–	Ghana Health Service
GSS	–	Ghana Statistical Service
HCl	–	Hydrochloric Acid
HRP	–	Histidine-Rich Protein
KCl	–	Potassium Chloride
Mbp	–	Mega Base Pair
MEGA	–	Molecular Evolutionary Genetics Analysis
MgCl	–	Magnesium Chloride
ML	–	Molecular Ladder
NCBI	–	National Center for Biotechnology Information
NH_4Cl	–	Ammonium Chloride
PCR	–	Polymerase Chain Reaction
PVP	–	Polyvinylpyrrolidone
RBCs	–	Red Blood Cells

RDT	–	Rapid Diagnostic Test
rRNA	–	Ribosomal Ribonucleic Acid
TBE	–	Tris-Boric EDTA
UDS	–	University for Development Studies
USAID	–	United States Agency for International Development
UV	–	Ultra-Violet
WHO	–	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is an infectious disease caused by species of the single-celled parasitic protozoan, *Plasmodium* (Cox, 2010; Da Silva et al., 2017; Bogitsh et al., 2019). The disease is a major public health and socio-economic problem in many countries, especially in sub-Saharan Africa (Gendrot et al., 2019). Five species of *Plasmodium* cause human malaria namely, *P. falciparum, P. malariae, P. ovale, P. vivax* and *P. knowlesi,* and these are transmitted through the bites of the female *Anopheles* mosquito (Cox, 2010; Taylor and Agbenyega, 2012; Amoah et al., 2019; Bogitsh et al., 2019; Thomson et al., 2019). *P. falciparum*, is by far the most virulent and prevalent of the five species in the West Africa sub-region (Adu-Gyasi et al., 2018). *P. vivax,* on the other hand, is the main aetiological agent of malaria outside the WHO-African region (WHO, 2018a).

In 2020, the World Health Organization (WHO) estimated global malaria cases to be around 241 million, resulting in about 627,000 deaths (WHO, 2021). In Ghana, the burden of malaria is heavy and is sustained by poverty (Awuah et al., 2018). An estimated 8 million malaria cases were recorded in the country in 2017 with 12,000 related deaths (WHO, 2018a). In response to the malaria threat, the WHO has initiated programmes aimed at reducing global malaria case incidence and mortality risk by 90 % by the year 2030 (WHO, 2015a).

Rapid and accurate diagnosis of malaria is an important step to effectively managing the disease (Gerstl et al., 2010; Berzosa et al., 2018). Detection of the *Plasmodium*

parasites in blood smears by microscopy is the gold standard for malaria diagnosis globally (WHO, 2015b), but this approach is expensive and comparatively sophisticated to use in limited resource settings (WHO, 2008). Similarly, more sensitive and efficient diagnostics such as nucleic acid detection methods such as the Polymerase Chain Reaction are expensive and even more sophisticated to use in field or resource-poor settings as is the case in many developing countries (Berzosa et al., 2018). Rapid Diagnostic Tests (RDTs) as a malaria diagnostic method are less sophisticated and have a short turnaround time (Adu-Gyasi et al., 2018). Malaria RDTs have been widely adopted globally since their introduction in the early 1990s (WHO, 2003, 2010a; Boyce and Meara, 2017; Cunningham et al., 2019) and over 200 different malaria RDTs commercially available for use (Gendrot et al., 2019).

In Ghana, as in most malaria endemic countries in Sub-Saharan Africa, the most available malaria RDTs detect the presence of *P. falciparum* histidine-rich protein 2/3 (*Pfhrp*2/3) antigens in the blood of humans (Amoah et al., 2016). Meanwhile, *Pfhrp*2/3 gene deletions have been reported in several African countries (WHO, 2019a) and Ghana is no exception, where some reports have detected between 11.1 % to 39.5 % deletion in confirmed *P. falciparum* isolates (Amoah et al., 2016; Ayelazuno, 2017; Amoah et al., 2020). The genetic variability of the Pfhrp2 target-marker has also been highly polymorphic in Ghana (Addai-Mensah et al., 2020). Variations in the parasite antigens targeted by RDTs, such as the lack or changes in targeted epitopes or the complete deletion of the gene in an isolate, as have been reported recently in some countries are particularly critical factors to the performance of many RDTs. The reported mutation in targeted proteins of the widely used RDTs such as the *Pfhrp2/3*

antigen is of greater concern to malaria diagnosis. Mutations to the *Pfhrp*2/3 antigen have serious implications for malaria diagnosis in Sub-Saharan Africa and impact the success of malaria intervention programmes in the region (Abukari et al., 2019; WHO, 2019a).

1.2 Problem Statement

Malaria RDTs are immunochromatographic lateral flow tests that work on the principle of monoclonal antibodies and detect antigenic proteins of *Plasmodium* species in human blood (Luchavez et al., 2011; Haberichter et al., 2017; Funwei et al., 2019). The diagnostic kits are less sophisticated, rapid and convenient to use. However, factors such as operator errors, low or very high parasitic load, coinfection with non-falciparum malaria species, absence of antigenic protein and/or flanking region are reported to impede the accuracy and efficiency of the test kits (WHO, 2019a). Most RDTs used in malaria diagnosis in Africa countries of detect the histidine-rich protein 2/3 antigens of *P. falciparum* (Amoah et al., 2016; Ugah et al., 2017). *Pfhrp*2/3 are inimitable and exclusively produced by *P. falciparum* (WHO, 2015a). Although *hrp2*-dependent RDTs are the most sensitive for *P. falciparum* malaria RDTs (WHO, 2020a), there are several reports of parasite strains that have mutations in *hrp*2-encoding genes or its similar *hrp*3 rendering it ineffective in such instances (Thomson et al., 2019; Nyataya et al., 2020; WHO, 2020a).

Amoah et al. (2016) reported a 33.3 % and 36.2 % *Pfhrp*2 gene deletion in microscopy and PCR-confirmed RDT positive isolates, respectively in Ghana. A recent study has also reported 12.9 % and 15.2 % exon 1-2 deletion of *Pfhrp2* and *Pfhrp3* genes,

respectively in Ghana (Amoah et al., 2020). The occurrence of mutations of *Pfhrp*2/3 genes raise questions about the reliability of *Pf*HRP2-RDTs for malaria diagnosis, especially in countries such as Ghana, where these kits are widely used (Bakari et al., 2020; King et al., 2021). This study sought to investigate the occurrence and prevalence of *Pfhrp*2 and 3 gene mutations in *P. falciparum* isolates in microscopy-confirmed blood samples from the Northern and Savannah Regions of Ghana.

1.3 Justification

Antigen-detecting RDTs are essential in the control of malaria in several parts of the world and thus, their availability and rate of use have dramatically increased over the last decade (WHO, 2016a, 2018b). In countries of Sub-Saharan Africa such as Ghana, *Pf*HRP2-RDT are widely employed due to the high prevalence of *P. falciparum* in these countries and exhibits good sensitivity in several field settings (WHO, 2019a). False negative RDT results have been linked to presence of *P. falciparum* with *Pfhrp*2 gene deletion (Amoah et al., 2016; WHO, 2019a). Several African countries have reported *Pfhrp*2 deletions and high diversity of the target gene in *P. falciparum* isolates (Baker et al., 2010; Amoah et al., 2016; Beshir et al., 2017; Bharti et al., 2017; Gupta et al., 2017; Kozycki et al., 2017; Parr et al., 2017; Nderu et al., 2019; Berzosa et al., 2020). However, in Ghana, extensive studies on the partial and full deletions and polymorphism of *Pfhrp2* gene among *P. falciparum* parasites are lacking (Addai-Mensah et al., 2020).

This study seeks to investigate the occurrence and prevalence of *Pfhrp*2 *and* 3 gene mutations in the *P. falciparum* isolates from the northern part of Ghana and appraise the

reliability of *Pf*HRP2-based RDTs for malaria diagnosis in the region. Data generated from this study will help inform policy on the most effective parasite-based diagnostic approaches to seek in medium and long-term planning.

1.4 Objectives of Study

1.4.1 Main Objective

The study seeks to assess the reliability of *Pf*HRP2-RDT in the diagnosis of malaria in northern Ghana by investigating the occurrence and prevalence of *Pfhrp*2 and/or *Pfhrp*3 gene mutations in *P. falciparum* isolates from microscopy-confirmed blood samples.

1.4.2 Specific Objectives

a. To evaluate the comparative performance of *Pf*HRP2-RDT and *P. falciparum*-specific PCR in detecting *P. falciparum* infection

b. To investigate the occurrence and prevalence of *Pfhrp*2 *and* 3 *gene* mutations in *P. falciparum* isolates from microscopy-confirmed malaria-infected blood samples in the Northern and Savannah Regions of Ghana

c. To assess genetic microvariations of *Pfhrp*2 and 3 genes in *P. falciparum* isolates from the Northern and Savannah Regions of Ghana.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria Infection

Malaria is an ancient disease whose history can be traced back to Chinese records as far back as 2700 BC (Cox, 2010). However, the discovery of bacteria in 1676 by Antonio Van Leeuwenhoek as well as the proposition of the germ theory by Louis Pasteur and Robert Koch between 1878 and 1879 were pivotal in the search for the real cause(s) of malaria (Bruce-Chwatt et al., 1988; Poser and Bruyn, 1999; Bortier, 2019). A breakthrough came in 1880 when a single-celled intracellular protozoan parasite was established as the cause of malaria by a French Physician, Charles Louis Alphonse Laveran (Harhangi et al., 1999). Malaria, from then, has been established as a female *Anopheles*-borne disease caused by the *Plasmodium* species.

2.2 Malaria Vectors and their Life Cycles

Human malaria is transmitted by the female of the *Anopheles* genus. Some of these malaria vectors that are widely distributed across sub-Saharan Africa include; *Anopheles gambiae, A. coluzzii, A. funestus* and *A. arabiensis* (Target Malaria, 2020; WHO, 2021). As in all mosquitoes, anophelines have two developmental phases: the egg to pupa and the pupa to adult phases (Morrow, 2007). The first phase comprises egg, larval and pupa stages that are exclusively aqua-zoned (Figure 2.1). The duration from the egg to the adult *Anopheles* varies between 7 days (in areas with ambient temperature around 31°C) and 20 days (in areas with the temperature of 20°C or less) (Warrell and Gilles, 2017).

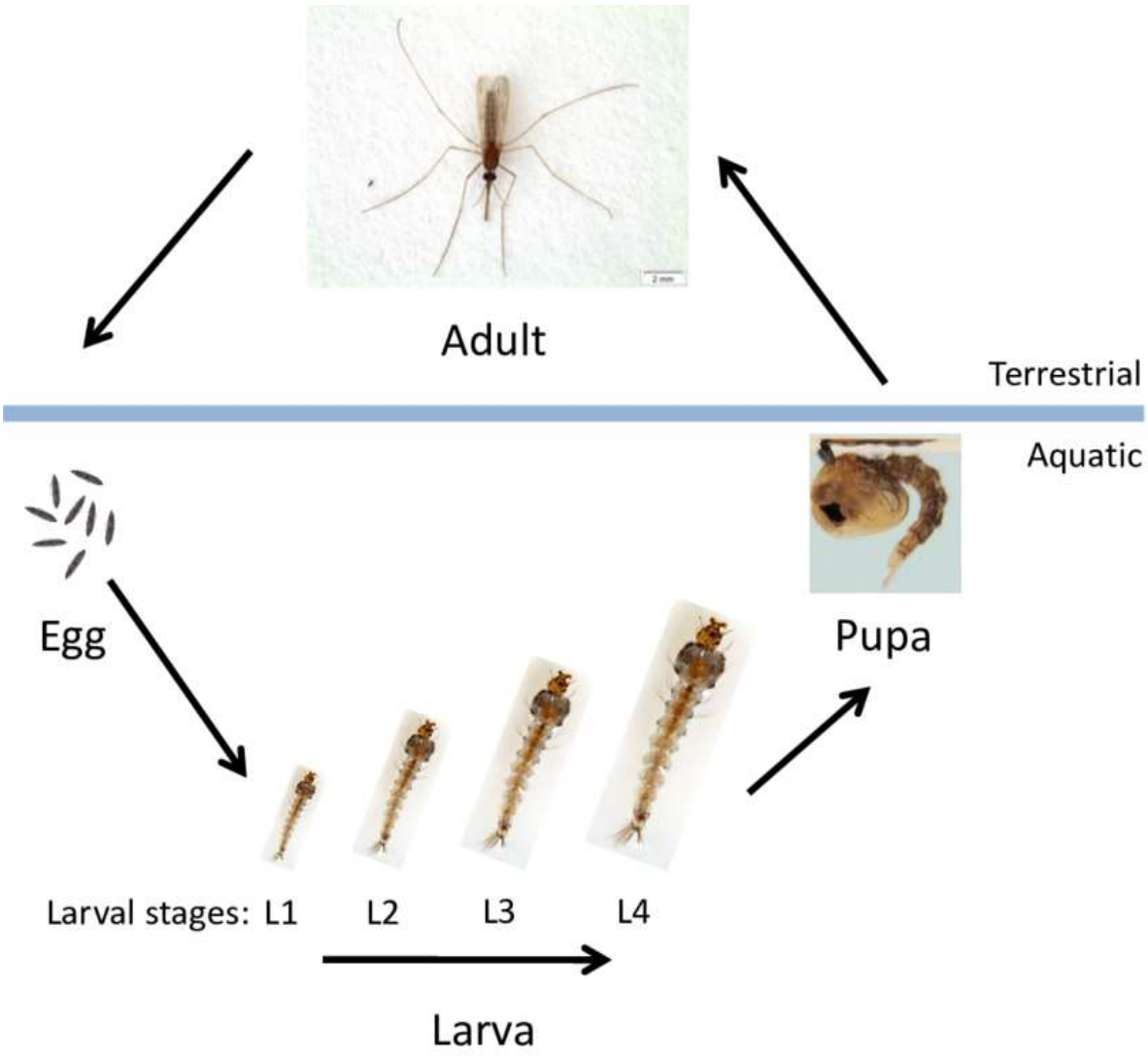


Figure 2.1: Life cycle of the *Anopheles* mosquito indicating various stages and breeding habitats

Source: USAID (2012)

2.2.1 The Ova Stage

The adult *Anopheles* female mosquito mates once in a lifetime and continually lays eggs. The mosquito uses blood to develop eggs (Gary et al., 2009). A day or two after a blood meal, an adult female *Anopheles* mosquito lays about 50 to 300 eggs in an oviposition

period. Eggs laid are about 0.5 x 0.2 mm in size (Figure. 2.2) and are laid directly on water including ponds, lakes, and stagnant water from rains and riversides. Depending on the ambient temperature, eggs may take about 7-14 days (around 16 °C) or 2-3 days (about 30 °C) to hatch into larvae (Gary et al., 2009).

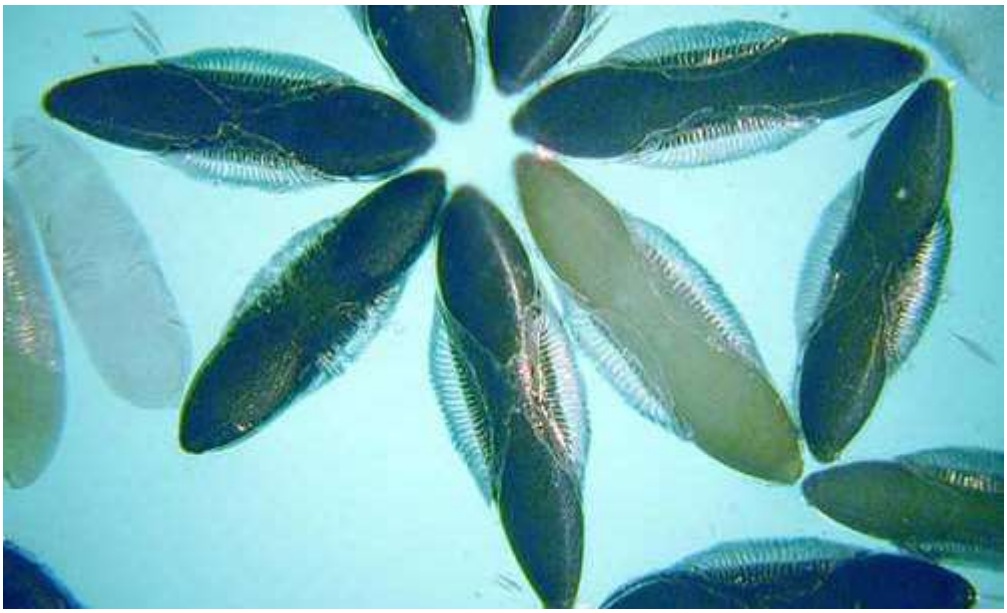


Figure 2.2: The ova of female *Anopheles* mosquito

Source: Rios and Roxanne (2007)

2.2.2 The Larval Stage

Each mosquito larva floats parallel to the surface of the aquatic habitat to breathe since it lacks a respiratory siphon. The mosquito larva develops a head (Figure 2.3) and mouth used mainly for feeding (USAID, 2012; Warrell and Gilles, 2017). The larvae feed on microorganisms (bacteria and algae) and other organic matter in their aquatic breeding habitat. The larva undergoes three moults leaping from 1 mm to 5 mm by the fourth stage (Figure 2.1) and has a relatively large thorax and abdomen of nine segments. In

normal tropical temperatures, the larvae take 5 – 10 days to develop into a pupa which is equally species-dependent (Warrell and Gilles, 2017).

Figure 2.3: Larva of the *Anopheles* mosquito showing its developed head and mouth brushes

Source: Rios and Roxanne (2007)

2.2.3 The Pupal Stage

When viewed from its sides, the pupa is morphologically seen as comma-shaped (Figure 2.4). It stays on the surface of the water to breathe through respiratory trumpets on its cephalothorax (Target Malaria, 2020). Although the pupa is motile and responds to stimuli, it does not feed at this developmental stage, also known as the resting or inactive stage. The pupal stage is also the stage of transformation from being aquatic to emerging and living out of water. In temperate zones, it takes 2 to 3 days for an adult mosquito to emerge from the dorsal surface of the cephalothorax (Coetzee et al., 2013)

Figure 2.4: The Pupal stage of mosquitoes

Source: Rios and Roxanne (2007)

2.2.4 The Adult Stage

Usually, the adult emerges from the pupa at dusk, rests for a short while for body hardening and goes for a flight in a large swarm if it is a male or flies into a swarm to mate if it is a female.

The morphological differences between a male and female *Anopheles* mosquitoes are the antennae and palps. Whereas the male has hairy antennae and long palps, the female possesses a smooth antennae and a regular palp. The Female *Anopheles* mosquitoes feed largely on blood to achieve fitness potential, increase their biting frequency and complete egg maturation processes (Gary et al., 2009; Barredo and DeGennaro, 2020). The male counterparts depend solely on nectar as food source for survival, rate of insemination and swarming ability (Gary et al., 2009; Ebrahimi et al., 2018).

There are about 430 adult *Anopheles* species worldwide, but only 62 transmit malaria across the globe (Target Malaria, 2020). About 47 of the 62 species are exclusive to the Americas, Eastern Mediterranean, Western Pacific and South-East Asia whereas 15 are specific to Africa (WHO, 2020c, 2021). However, *A. gambiae, A. arabiensis, A. funestus, A. pharoensis* and *A. nili* are widely distributed across the sub-Saharan Africa (WHO, 2020c, 2021). Other *Plasmodium* vectors in Africa include *A. coluzzii, A. hispaniola, A. labranchiae, A. melas, A. moucheti, A. multicolor, A. sergentii, A. leesoni, A. rivulorum, A. stephensi and A. vaneedeni*. *Anopheles gambiae s.l.* and *A. funestus* and these are major malaria vectors in Northern Sahel, middle transitional and Southern ecological zones of Ghana (GHS, 2014a). *Anopheles arabiensis* has been found in Sahel zone but in fewer numbers (GHS, 2014a).

2.3 Aetiological Agents of Human Malaria

Presently, there are five *Plasmodium* species known to cause human malaria worldwide (WHO, 2019b), namely, *Plasmodium malariae, Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and lately Plasmodium *knowlesi* which was originally only a simian parasite (WHO, 2014). Some of these species are reported to be geographically specific. However, the female *Anopheles* mosquito is the common vector of economic importance to all the *Plasmodium* species that cause malaria (Plucinski et al., 2019).

2.3.1 *Plasmodium malariae*

The first parasite identified as a cause of human malaria was *P. malariae* which Charles Louis Alphonse Laveran described in 1880 (Collins and Jeffery, 2007; Cox, 2010; Symington, 2012; Bogitsh et al., 2019) and its genome was sequenced in 2017 (Rutledge

et al., 2017). Malaria caused by *P. malariae* is termed quartan malaria because the cyclic lysis of red blood cells (RBCs) is caused by the development of the parasite occurs every 3−4 days. This species of *Plasmodium* also has the longest incubation period (18−40 days) (Brasil et al., 2011). Although *P. malariae* has no dormant or hypnozoites stage, infected patients could have prolonged erythrocyte infection. The erythrocyte infection may be asymptomatic and later emerge symptomatically months to years later when patient had even left an endemic area. *P. malariae* has been reported in all WHO global administrative regions (WHO, 2019b). Morphologically, *P. malariae* are compact parasites at all stages and merozoites in rosette without altering host's erythrocyte, and produce about 8−10 merozoites per schizont (WHO, 2014; Warrell and Gilles, 2017).

2.3.2 *Plasmodium falciparum*

P. falciparum is one of the causative parasites of malaria and causes a form of the disease called malignant or *falciparum* malaria. William Welch originally described *P. falciparum* in 1897 (Bogitsh et al., 2019). The severity of malignant malaria is attributed to the rapid multiplication of *P. falciparum* sporozoites (blood stage parasites) in human blood which could leads to severe anaemia by haemolysis and alters the surfaces of infected RBCs (Berhane et al., 2018; Zekar and Sharman, 2021). The *Plasmodium* species causes fatal human malaria and produces the highest rates of malaria complications, morbidity and mortality (WHO, 2020b). The 2019 World Malaria Report attributed about 90 % of the world's malaria mortality to *P. falciparum,* making it an important threat to global public health (WHO, 2019b). In 2018, *P. falciparum* accounted for 99.7 % of malaria parasites in the African region, 71 % Eastern

Mediterranean region, 65 % in Western Pacific, and 50 % in Southeast Asia (WHO, 2019b).

P. falciparum has an average incubation period of about 11 days, the shortest among *Plasmodium* species (Geraldo et al., 2017; Jensen et al., 2019). The morphology of *P. falciparum* is not fixed as it undergoes changes in the course of its life cycle (Sato, 2021). The length of its sporozoites is between 10 and 15 µm long and spindle-shaped, has thick pellicles with pointed ends of equal size. The single nucleated sporozoites adapt their peripheral fibres for locomotion (Warrell and Gilles, 2017). The ovoid schizont that develops from sporozoites after it infects liver cells is about 30-70 µm in diameter and would later develop in merozoites which are ~1.5 µm and 1 µm in length and diameter, respectively (Warrell and Gilles, 2017; Bogitsh et al., 2019; Schlabe et al., 2021).

The genome of *P. falciparum* was sequenced (~23.3 Mbp) in 2002 with about 5,403 described genes organized into 14 chromosomes (Gardner et al., 2002; Oyola et al., 2016; Nag et al., 2018). Variation within the genetic make-up of the parasite is fundamental to its pathogenesis, especially when variation of alleles in different clones is believed to aid immune evasion (Biggs et al., 1991). This attribute of the parasite poses a significant challenge to the development of malaria vaccines even though good progress has been made thus far in this regard (Richie and Saul, 2002). The latest inventory (PlasmoDB rel. 9.0) genomic data of *P. falciparum* on the PlasmoDB database (https://www.plasmodb.org) indicates 5,720 coding genes with a cumulative nucleotide length of 22.3 Mbp. Variations have been identified in several stages of *P.*

falciparum isolates (Moser et al., 2020). Some markers under study include the two dominant proteins known to challenge the human system; merozoite surface proteins 1 and 2, glutamate-rich protein (GLURP) and circumsporozoite protein (Metoh et al., 2020). The genomic variation of *P. falciparum* has led to the discovery and clones of several strains of the species that are used in epidemiological and population genetic studies. Some of these strains include 3D7 (wild type), NF166 (Guinea), 7G8 (Brazil), Dd2 (Thailand), KH (Cambodia) among others (Moser et al., 2020).

2.3.3 *Plasmodium vivax*

Plasmodium vivax was first described by Giovanni Battista Grassi and Raimondo Feletti in 1890. Among the five human malarial parasites, *P. vivax* has the widest geographical reach, surviving and thriving in tropics, subtropics and temperate climatic areas (Warrell and Gilles, 2017). Though *Plasmodium vivax* is globally distributed, it is more prevalent in Latin America and Southeast Asia (WHO, 2015b, 2020d).

According to the 2019 Global Malaria Report, 53 % of the global *P. vivax* infections was reported in Southeast Asia, with India being the most burdened in the region (47 %) (WHO, 2019b). In the South America, *P. vivax* was responsible for up to 75 % of malaria cases in 2019, earmarking it as the predominant parasite in that region (WHO, 2019b). It also accounted for 29 % of malaria cases in the Eastern Mediterranean in the same year (WHO, 2019b).

For a long period, *P. vivax* was regarded as benign but has lately been implicated as a cause of malaria cases that leads to high fatality rates (Tjitra et al., 2008; Mahgoub et al., 2012; Abdelwhab et al., 2021;). *Plasmodium vivax* has a 12−17 days incubation

period, but sporozoites can remain dormant in the liver for 9−12 months or longer before surfacing as schizonts to cause infection (Brasil et al., 2011). The distinctive characteristics of *P. vivax* from *P. malariae, P. falciparum* and *P. knowlesi* is its ability to enlarge infected erythrocyte, presence of granules known as Schiifner's dots and ameboid trophozoite (Taylor and Agbenyega, 2012; Warrell and Gilles, 2017).

2.3.4 *Plasmodium ovale*

P. ovale is a rare *Plasmodium* species that cause less fatal malaria in humans. It was originally described by John William Watson Stephens in 1922 (Stephens, 1922; Bogitsh et al., 2019). Incidences of *P. ovale* have been recorded in about 107 countries in Africa and Asia. The parasite, however, is endemic to tropical western Africa (Snounou et al., 1993; Baird and Hoffman, 2004; Fuehrer et al., 2010; Li et al., 2013). *P. ovale* has two morphologically similar but genetically distinct subspecies namely, *P. ovale curtisi* and *P. ovale wallikeri* and has an incubation period of 13−14 days (Win et al., 2004; Oguike et al., 2011; Mendelson, 2013; Fuehrer and Noedl, 2014; Miller et al., 2015). The biological and morphological adaptation of *P. ovale* is very similar to *P. vivax* as they enlarge infected erythrocyte, has Schiifner's dots and ameboid trophozoite as distinct mutual features (Dinko et al., 2013). However, *P. vivax* has some additional morphological features at various stages of the parasites, which include trophozoite compactness, fewer merozoites in schizont and elongated infected erythrocytes of hosts (Warrell and Gilles, 2017).

2.3.5 *Plasmodium knowlesi*

The morphology of *P. knowlesi* was first described by Das Gupta and Robert Knowles in 1932 (Ngernna et al., 2019). *P. knowlesi* was long known to be a causative *Plasmodium s*pecies of simian malaria where *Macaca fascicularis* ('kra' monkey) served as a natural vertebrate host (Kantele and Jokiranta, 2011; WHO, 2017). Currently, the parasite is recognized as the fifth *Plasmodium* species that cause malaria in humans after several cases were reported in Malaysia in 2004 after the first case in 1965 in Southeast Asia (Singh et al., 2004; Antinori et al., 2013; Amir et al., 2018). It is now described as a zoonotic malaria parasite (WHO, 2017), with cases recorded in nearly all Southeast Asian countries where it is deemed endemic (WHO, 2020c).

P. knowlesi has the shortest asexual replication cycle, which leads to rapid increases in parasitemia, making its infection mostly chronic, symptomatic and sometimes fatal (WHO, 2016b). Despite the relatedness with other malarial parasites, *P. knowlesi* has some distinctive phenotypic variances which includes cell preference of host cell, length of asexual cycle and the absence of a dormant liver stage (hypnozoite) (Amir et al., 2018).

The genome of *P. knowlesi* was sequenced in 2008, and is recorded to be 23.5 Mbp containing ~5,185 genes in 14 chromosomes (Pain et al., 2008).

2.4 Life Cycle of *Plasmodium* Species

The life cycle of the *Plasmodium* comprises several distinct stages in the insect (female *Anopheles* mosquito) and vertebrate (human) hosts (Figure 2.5). This consist of an asexual stage and a sexual stage. The asexual cycle begins when an infected female

Anopheles mosquito injects sporozoites into the human host while feeding (Grant, 2008). The sporozoites find their way into the bloodstream and are carried to the liver, infecting the hepatic cells (Grant, 2008; Jensen et al., 2019). In the liver cells, sporozoites develop into schizonts and later rupture, releasing numerous merozoites back into the bloodstream (Warrell and Gilles, 2017). Rupture of the schizont, also known as hepatic schizogony, is said to be the likely source of fever associated with malaria (Taylor and Agbenyega, 2012) In the case of *P. vivax* and *P. ovale*, some parasites may remain dormant in the liver cells for months to years. The dormant stage of the parasites in the host is known as hypnozoite (WHO, 2014; Warrell and Gilles, 2017).

The released merozoites infect red blood cells, grow into a ring-shaped form, and subsequently enlarge to form the trophozoite. Each trophozoite later develops into a schizont at maturation and repeatedly divides to produce many new merozoites. Merozoites rupture the infected RBC, and travel through the bloodstream to infect other RBCs (Bogitsh et al., 2019). These infective stages are repeated cyclically in human blood, thus causing illness and death if not treated (Bogitsh et al., 2019). As some merozoites keep to the replicative cycle, other merozoites differentiate into male or female sexual forms called gametocytes upon infecting RBCs (Warrell and Gilles, 2017). However, the gametocytes do not cause illness to the vertebrate host but are infective to the *Anopheles* mosquito. When a mosquito takes bloodmeal from an infected human, they suck circulating gametocytes (male and female) that infect the insect host (Willie, 2018). The cycling duration in the vertebrate hosts is *Plasmodium*

species-dependent (Ayelazuno, 2017). For instance, the hepatic schizogony stage of the cycle takes ~5 days for *P. falciparum* and ~15 days for *P. malariae* (Warrell and Gilles, 2017).

The sexual stage of infection occurs in a mosquito and begins when a suitable *Anopheles* species ingests gametocytes. Gametocytes from the infected blood cells become gametes as male and female gametes fuse to form a zygote (Bogitsh et al., 2019). In about 15 hours, the zygotes slowly elongate into a motile ookinete and move through the outer stomach wall. The ookinete develops into a non-motile oocyst between 24 and 72 hours after the blood meal matures and ruptures to release sporozoites upon oocyst maturation (Taylor and Agbenyega, 2012). These sporozoites migrate to the salivary glands of the insect, ready to be injected at its next bloodmeal to begin another asexual stage. The Anopheles mosquito's distinct stages can span 10 days (Warrell and Gilles, 2017).

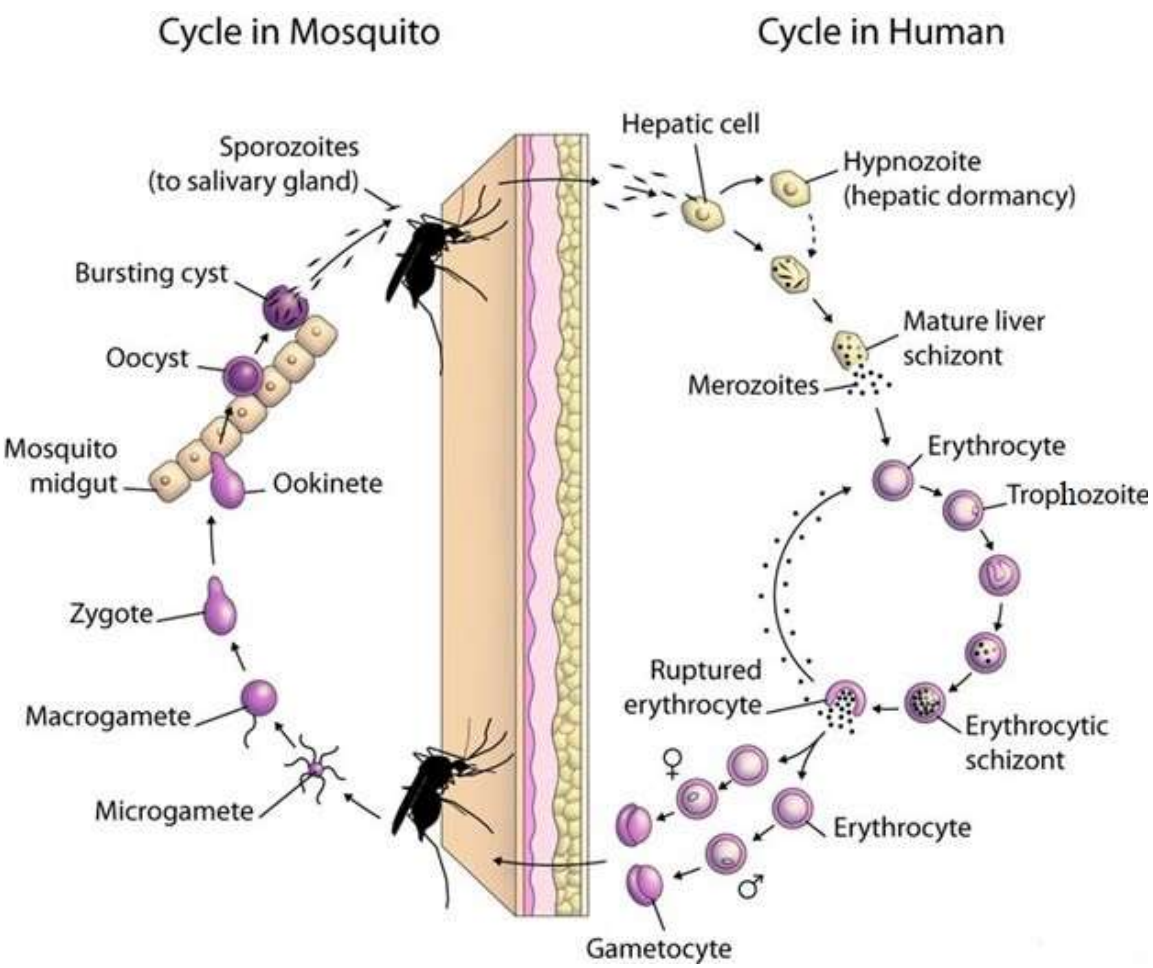


Figure 2.5: A descriptive life cycle of human *Plasmodium* species in a two-host system

Source: University of Thessaly and European Union (2013)

2.5 Clinical Symptoms of Malaria Infection

Symptoms of malaria may be specific or non-specific manifest differently in different individuals, and completely asymptomatic or mild in immune individuals (WHO, 2014). Clinical symptoms mostly result from the asexual stages of the parasite in human blood (Zekar and Sharman, 2021). All clinical symptoms of malaria are caused by the invasion of the erythrocytes by merozoites and their multiplication in the host RBC (Jensen et al., 2019). Symptoms of uncomplicated malaria generally include fever, chills (feeling

unusually cold), rigors (shivering) and headache. Other clinical symptoms of malaria may include generalized body and joint pain, nausea and/or vomiting, profuse sweating, bitterness in the mouth, irritability and lack of appetite, especially in infants (GHS, 2014a).

Clinical symptoms of malaria may happen in discrete or several symptoms can present at a time (WHO, 2015b). Generally, malaria's main symptoms may resemble those presented by diseases such as pneumonia, meningitis, enteric fever or septicemia (GHS, 2014a; Bogitsh et al., 2019).

2.6 Economic Impact of Malaria

Plasmodium species are medically important as agents of malaria (Sato, 2021), which is undoubtedly one of the most devastating diseases ever to affect humans (Mba and Aboh, 2004; Amoah et al., 2019; Sato, 2021). Although malaria can be prevented or treated, the disease still affects 232 out of every 1,000 persons at risk of the disease in the global population (WHO, 2021). Besides malaria's health burden the disease also poses economic challenges on families and nations (WHO, 2020b).

A WHO report about Africa revealed that malaria alone accounts for about 79.5 % of productivity losses due to parasitic and vector-borne diseases (WHO, 2019c). Productivity losses include costs of healthcare, absenteeism at work and/or school, brain damage caused by cerebral malaria, and loss of investment and tourism revenues (Chima et al., 2003; Hong, 2011; WHO, 2019c). Children, who are the most vulnerable to malaria (WHO, 2018a), are more likely to learn less at school since they are frequently absent or unable to concentrate due to illness (Kihara et al., 2006). In

addition, malaria in the working population in malaria-endemic countries decreases production due to illness or caring for ill children and may cause a decline in wages and savings (Sauerborn et al., 1996; Kihara et al., 2006; Hong, 2011).

2.7 Epidemiology of Malaria

2.7.1 Global Distribution

Malaria remains a life-threatening disease (WHO, 2018a, 2019c),with an estimated 241 million malaria cases recorded in 2020 in 85 global malaria-endemic countries (WHO, 2021) The 241 million cases were a 5.8 % increase from the 2019 records that was partly attributed to partials negligent of malaria control strategies due to the COVID-19 pandemic (WHO, 2019b, 2020d). In 2000, global malaria case incidence stood at 81 per 1000 persons at risk which is significantly lower than 59 per 1000 of the population at risk recorded in 2015 and 56 per 1000 persons at risk in 2019 (WHO, 2015c, 2019c, 2020d). While records indicate a decline in malaria incidence over the last decades, the incidence of malaria at 59 per 1000 of the population at risk in 2020 was significantly higher than that recorded in 2019 (WHO, 2019b). This recent increase in incidence has also been attributed to the disruption of malaria control programmes at the peak of the COVID-19 pandemic worldwide (WHO, 2021).

In 2000, there were 896,000 global malaria-related deaths compared to 562,000 deaths in 2015 (WHO, 2015c; Cibulskis et al., 2016; WHO, 2021). In 2019 and 2020, malaria-related deaths recorded were 558,000 and 627,000, respectively (WHO, 2020c, 2021). The South-East Asia region of the WHO has 1.64 billion persons at risk (Table 2.1) of human malaria (WHO, 2021). Despite the global impact of malaria, some regions or

countries are malaria-free. These are all WHO European countries, El-Salvador, Belize, Argentina, Paraguay, China, Timor-Leste, Sri Lanka, Maldives, Timor-Leste and Morocco (WHO, 2015c, 2021).

2.7.2 Distribution in WHO Africa Region

Africa is the most malaria-burdened WHO region in the world (WHO, 2020c). Transmission of Malaria in the African region is all year-round except for Algeria and Cape Verde, and almost exclusively due to *P. falciparum* (WHO, 2014) as shown in Table 2.1. Out of the estimated 241 million global malaria cases recorded in 2020, Africa accounted for approximately 95 % (228 million) cases (WHO, 2020c). Six African countries, including Nigeria, DR. Congo, Uganda, Mozambique, Angola and Burkina Faso, accounted for 55 % of all global malaria cases in 2020 (WHO, 2021). The malaria case prevalence per 1000 of the population at risk in Africa stood at 368 cases and 222 cases in 2000 and 2019, respectively, indicating a significant reduction in malaria cases at the dawn of the 21st century. In 2020 the case report stood at 232, which WHO attributes mainly to disruptions to malaria control measures during the COVID-19 pandemic (WHO, 2019b, 2020d).

With regards to malaria deaths, the general trend of reduction in deaths shown by a decline from 680, 000 deaths in 2000 to 384, 000 deaths in 2019 but was also upset by the record of 602, 000 deaths in 2020 (WHO, 2020c). This increase in mortality in 2020 has also been attributed to the effects of COVID-19 on malaria control strategies (WHO, 2021). It is worth mentioning that even though the impact of Malaria in Africa, especially sub-Saharan Africa is huge, Cape Verde and Sao Tome and Principe have not

recorded malaria cases since 2018. Algeria has also been certified malaria-free since May 2019 (WHO, 2020c).

Table 2.1: Global malaria distribution and prevalent *Plasmodium* species per WHO administrative regions

WHO Region	**Population at Risk**	**Prevalent *Plasmodium* spp.**	**Estimated Cases (Deaths)**		
			2010	**2015**	**2019**
Americas	139 million	*Pv* – 76 %	821,000	561,000	889,000
		Pf and mixed – 24 %	(510)	(400)	(550)
Eastern Mediterranean	324 million	*Pf* and mixed – 73 %	5 million	4.1 million	5.2 million
		Pv – 27 %	(8,750)	(7,880)	(10,130)
Western Pacific	767 million	*Pf* and mixed – 68 %	1.8 million	1.4 million	1.7 million
		Pv – *32* %	(3,780)	(2,780)	(3,160)
South-East Asia	1.64 billion	*Pf* and mixed – 53 %	24.6 million	13.3 million	6.3 million
		Pv – 46 %	(2,421)	(620)	(162)
Africa	139 million	*Pf* – 98 %	215.4 million	199.1 million	214.6 million
		Pv and others – 2 %	(541,500)	(417,600)	(384,469)

Pf – Plasmodium falciparum; *Pv – Plasmodium vivax*; Other *Plasmodium* spp. is <1 % across all region

2.7.1 Epidemiology of Malaria in Ghana

In Ghana, malaria is hyper endemic and has dominated outpatient morbidity with 30.4 million of the population being at risk of the disease (GHS, 2007, 2018; WHO, 2020c). Malaria incidence is year-round in Northern Sahel, middle transitional and Southern ecological zones of Ghana with *Anopheles gambiae s.l.* and *A. funestus* being the major malaria vectors (GHS, 2014a). Between the years 2002 and 2017, malaria constituted 20.30 to 48.22 % of total out-patient cases (Table 2.2) recorded by the Ghana Health Service (GHS, 2002, 2014a). Between 2000 and 2019, there has been about 290,000 malaria related deaths in Ghana (WHO, 2020c) and accounted for most OPD mortalities.

Table 2.2: Malaria Outpatient (OPD) morbidity numbers recorded in health facilities in Ghana

Year	No. of Reported Cases	Malaria Percentage of Total OPD cases (%)
2002	3,169,949	44.06
2003	3,417,304	44.69
2004	3,453,806	45.09
2005	3,799,158	44.76
2006	3,948,078	38.70
2007	5,499,262	43.41
2008	5,130,427	48.22
2009	7,096,440	46.2
2010	8,208,670	43.9
2011	1,0171,448	40.0
2012	10,171,448	39.98
2013	10,839,392	34.71
2015	7,598,999	28.45
2016	6,959,525	31.1
2017	6,133,302	20.3

2.8 Prevention and Control of Malaria

Control of malaria involves a cohesive approach comprising vector control, chemoprophylaxis, rapid, and effective treatment (WHO, 2015b). The production and full administration of an effective malaria vaccine is the ultimate goal in eliminating

global mortality and morbidity (Feachem and Sabot, 2008). Unfortunately, to date, the search is still on to develop this vaccine. The RTS, S (Mosquirix ™) malaria vaccine has been the only vaccine to meet some of the standards set for an effective malaria vaccine. And thus, as of 2021, more than 2.3 million RTS, S malaria vaccine have been administered in the final piloting phase after it has passed all stages of vaccine trials (WHO, 2021). However, the vaccine has been reported to have only modest efficacy, especially at high malaria transmission sites and also offers different protection in different age groups. While there is still hope for an effective malaria vaccine in the near future, the use of a cohesive approach remains a priority in malaria-endemic regions worldwide.

Vector control breaks the transmission cycle by preventing infected *Anopheles* mosquitoes from transmitting malaria parasite. Interventions employed to control the malaria vector include the use of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS) (Ayelazuno, 2017).

2.8.1 Insecticide-treated mosquito nets (ITNs)

Insecticide-treated bed nets provides an effective barrier between the person sleeping under it and mosquito vector (USAID, 2012). It purposely repels or kill mosquitoes and/or other insects, reducing the number of mosquitoes feeding on persons (Ayelazuno, 2017). The WHO reports that almost 2.2 billion ITNs were distributed globally between 2004 and 2019 (WHO, 2020c). Out of this, 86 % (1.9 billion) were distributed to only sub-Saharan Africa (WHO, 2020c). As of 2019, 78.60 % of Ghana's population had access to an insecticide-treated net (WHO, 2020d).

2.8.2 Indoor residual spraying (IRS)

Indoor residual spraying involves spraying inner walls of houses with WHO approved insecticides with residual properties (USAID, 2012). It is one of several malaria vector control interventions strategies adopted by many malaria endemic countries including Ghana. The IRS repels mosquitoes from entering the place of residence and kills female *Anopheles* mosquitoes that rest inside the house after their blood meal (Sharp et al., 2007; Kim et al., 2012). In settings where malaria transmission persists for three (3) months and insecticides are efficient for at least three (3) months, the spray cycle is once a year, but where malaria transmission is year-round, at least two rounds of spraying in a year is recommended (WHO, 2015d). The WHO recommends IRS utilization alone or in combination with other interventions such as ITNs (WHO, 2018a), especially in endemic and hyperendemic settings (Plues et al., 2010).

The percentage of populations at risk that are being protected by IRS in countries was recorded to have declined from 5 % in 2010 to 2 % in 2019 in every WHO region (WHO, 2020c). In Ghana, IRS was first piloted by AngloGold Ashanti through its malaria programme (AGAMal) as part of its cooperate social responsibility in the Obuasi Municipality of Ghana in 2006 (Suuron et al., 2020). Currently, it is practiced nationwide and championed by the national malaria control programme of Ghana.

2.8.3 Chemotherapy

Chemoprophylaxis is a therapeutic mechanism of preventive medications against malaria transmission and infection using antimalarial drugs (WHO, 2019b, 2022). Chemoprophylaxis usually targets non-resident travellers to endemic regions, pregnant

and breastfeeding mothers and non-immune in and/or travelling to areas where there is a medium to high risk of malaria infection, i.e., >10 cases per 1000 inhabitants per year (Schlagenhauf and Petersen, 2008; Moehrle, 2022). Recommended chemoprophylaxis drugs include mefloquine, doxycycline and atovaquone/proguanil (Petersen, 2004; Moehrle, 2022; WHO, 2022).

2.9 Treatment of malaria

Malaria is treatable, and prompt antimalarial treatment saves many lives yearly (WHO, 2012). Appropriate treatment of uncomplicated malaria is crucial in minimizing the development of severe disease and lowering malaria transmission in a community (WHO, 2012). For many decades, several natural and synthetic compounds have been employed as chemotherapy for malaria (Ginsburg and Deharo, 2011). These include chloroquine, quinine sulphate, artemisinin, mefloquine, hydroxychloroquine, furoquinolines, cryptolepines, mepacrine and halofantrine (White, 2004; Tse et al., 2019). Due to the development of resistance, some of these compounds such as chloroquine, quinine and mepacrine are no longer in used as singular compounds (Onguéné et al., 2013; Tse et al., 2019). Artemisinin was previously used as a mono-therapeutic drug against human malaria. Due to the reported emergence of resistance to the drug, the Artemisinin-based Combination Therapy (ACT) is now recommended globally by the WHO as the first-line drug for the treatment of uncomplicated malaria (White, 2004). From 2004, ACT was adopted nationally for the treatment of uncomplicated malaria in Ghana (GHS, 2014a). The 2014 revised treatment policy selected three (3) ACTs for use in Ghana and has been in force ever since (GHS, 2014b). These are Artesunate-Amodiaquine (AS-AQ), Artemether-Lumefantrine (A-L) and

Dihydroartemisinin-piperaquine (DHAP). Aside A-L, which is not used in children who are less than six months of age, all three are safe for the treatment of malaria in children and adults in Ghana (GHS, 2014a).

2.10 Diagnoses of Malaria

The precise and timely diagnosis of malaria are important in the management, control and surveillance of the disease as clinical symptoms are non-specific and could be similar to symptoms of other diseases (Berzosa et al., 2018). Malaria misdiagnosis may expose patients to side effects of drugs, heighten the incidence of antimalarial resistance, and cause health professionals to overlook fatal illnesses such as pneumonia, Lassa fever, Ebola and Middle East respiratory syndrome, which present similar symptoms as malaria (Grandesso et al., 2016). For this and other reasons, the WHO recommends confirmation of all clinical suspicion of malaria by diagnostic tests before treatment (WHO, 2009). Several techniques are employed for malaria diagnosis, parasite quantification and/or species differentiation. These include microscopy, rapid diagnostic tests (RDTs) and Polymerase Chain Reaction (PCR) each if which comes with its strengths and limitations.

2.10.1 Light Microscopy

The microscopic examination of thick and thin blood smears stained in 10 % Giemsa is the gold standard in malaria diagnosis, quantification and speciation (Bailey et al., 2013). This technique is relatively inexpensive compared to other techniques for malaria diagnoses (Singh et al., 1999). However, accurate identification and speciation hinge on factors such as the experience of microscopists, proper staining of microscopic slides,

turnaround time and electricity supply. The sensitivity and specificity of microscopy are 50 to 500 parasites/µl (Moody, 2002; Berzosa et al., 2018) when used by well-trained personnel under field conditions. This is, however and reduced by the increasing the experience of microscopists (WHO, 2015e; Bortier, 2019). Microscopy is the preferred technique for diagnosis of severe malaria as it assesses important prognostic parameters such as parasitic load, development stage of the parasite and intra-leukocyte pigmentation (WHO, 2015e).

2.10.2 Rapid Diagnostic Test (RDT)

The Rapid Diagnostic Test (RDT) is an immunochromatography method relying on the mechanism of monoclonal antibodies (mAbs) to detect parasite antigens in blood samples of persons infected with *Plasmodium* (Luchavez et al., 2011; Mouatcho and Goldring, 2013; Herman et al., 2019). Malaria RDTs are generally made up of a nitrocellulose strip that is inserted in a plastic cassette but they can also come in the form of a dipstick which is a self-standing strip that is dipped in a tube, or they can come in the form is described as the cardboard format (Maltha et al., 2013). In this diagnostic technique, parasitic antigens in a peripheral blood sample collected from a finger-prick are detected by prepared mAbs immobilized on the RDT kit (Moody, 2002).

RDT kits have been an important alternative to other malaria diagnostic tools since their introduction into the healthcare system (WHO, 2011). These RDTs are relatively less sophisticated with a fast turnaround time and do not require complex equipment or electricity. They are therefore advantageous for use in peripheral health facilities (Roh

et al., 2016). However, RDTs cannot be used to quantify parasite load (Haberichter et al., 2017).

The different RDTs widely used across the globe produce different assessments of sensitivity and specificity, which may be broadly attributed to the manufacturing process (WHO, 2012). Nonetheless, reduced RDT sensitivities and specificities that users commonly report may also be due to parasite and operator factors such as persistent antigen from recent past infection, immunologic cross-reaction, operator errors, antibody degradation due to storage and transport condition, lack or low expressible levels of target antigen, variation in amino acid residues of targeted epitopes or, flooding of excess antigens or antibodies (Cnops et al., 2011; Luchavez et al., 2011; Grandesso et al., 2016; Dalrymple et al., 2018). Malaria RDTs are generally convenient and have sensitivities of approximately 100 parasites/µl (Berzosa et al., 2018; WHO, 2020b).

Antigen targets of malaria RDTs may be specific to a particular *Plasmodium* species or several *Plasmodium* species that cause human malaria (WHO, 2015b). Most approved malaria RDTs that are currently in use targets three (3) antigens namely, the histidine rich-protein 2/3 (HRP*2/3*), parasite lactate dehydrogenase (pLDH) and parasite aldolase (pAldo) (Table 2.3). Different characteristic of the presented antigens may affect their suitability for use in different clinical cases (WHO, 2015b).

Table 2.3: Target *Plasmodium* antigens of current commercial RDT kits

Target antigen	**Detecting Human *Plasmodium* species**
HRP2/3	*P. falciparum*
Pf-pLDH	*P. falciparum*
P. vivax-pLDH	*P. vivax*
Pan-pLDH	All human *Plasmodium* spp.
Parasite aldolase (pAldo)	All human *Plasmodium* spp.

The HRP2 protein was identified in 1974 in *Plasmodium lophurae,* an avian parasite (Kilejian, 1974). Since then, other three unique homologues of this protein have been discovered exclusively in the genome of *P. falciparum* out of the five *Plasmodium* species that infect humans (Poti et al., 2020). These include histidine rich protein 1 (HRP1), HRP2 and HRP3 proteins. For this reason, the detection of these proteins in infected human blood only confirms *P. falciparum* malaria (Grandesso et al., 2016). The *hrp*2 gene is 85−90 % homologous to *hrp*3 in terms of nucleotide sequence (Poti et al., 2020) with numerous translated epitope repeats comprising 34 %, 37 % and 10 % of the amino acid histidine, alanine and aspartic acid, respectively, found in both proteins. The detection of *Pf*HRP2 or its cross-reaction homologue *Pf*HRP3 has served as a target protein for several brands of malaria RDTs over the years exhibiting good sensitivity in several field settings (WHO, 2019a).

The pLDH protein, an intracellular metabolic enzyme, is also commonly target in several brands of malaria RDTs. This protein is expressed by all *Plasmodium* species

making it a universal antigen for detecting all *Plasmodium* species infection and is produced during the sexual and asexual stages of the *Plasmodium* species (Gerstl et al., 2010). An important advantage of pLDH antigen as an RDT target in comparison to *Pf*HRP2-RDT is its rapid elimination from the blood stream after treatment (Alemayehu et al., 2020). This attribute eliminates the occurrence of false positive by persistent antigen which is a limitation to *Pf*HRP2-RDT (Grandesso et al., 2016). However, pLDH kits are less sensitive than *hrp2* tests (Alemayehu et al., 2020). The LDH amino acid array vary between *P. falciparum* and other *Plasmodium* species, allowing for the production of species-specific LDH diagnostic kits (Li et al., 2017).

2.10.3 Molecular-based diagnostics

Molecular-based malaria diagnostics techniques are useful for population surveys, focus research on malaria elimination and management schemes, and molecular characterization of malaria parasites (Strøm et al., 2014; Britton et al., 2016). These techniques are also valuable for drug resistance studies and other specialized epidemiological research but are generally unavailable for large-scale field use in endemic areas because of high cost (WHO, 2020d). Currently, molecular-based nucleic-acid amplification methods such as PCR have a limited role in the clinical management of malaria, especially in malaria-endemic countries (WHO, 2015b, 2015c). PCR and loop-mediated isothermal amplification, for instance, are, however highly sensitive and very useful for detecting mixed parasitic species infection, especially at low parasite load that is undetectable by light microscope or RDTs (Berzosa et al., 2018).

PCR and its variations in conventional, nested, or real-time PCR can detect parasite DNA in parasite concentrations as low as 1-5 parasites/µl of peripheral blood (Britton et al., 2016; Berzosa et al., 2018). Despite their superior sensitivity and specificity in malaria diagnosis, the routine use of PCR and other molecular diagnostic techniques is very limited in low to medium income countries. This is attributable to the high cost of usage and need for trained personnel making it impossible for adoption in low to medium income countries (Mens et al., 2007).

2.11 Major Epitopes of *Plasmodium falciparum* histidine-rich protein 2/3 RDTs

The genes that encode *Pf*HRP 2 and 3 proteins in the *Plasmodium falciparum* are found in labile sub-telomeric regions of chromosomes 8 and 13, respectively. These regions contain two exons each but one intron as in the case of *Pfhrp*2 or two introns as in the case of *Pfhrp3* (Abdallah et al., 2015; Addai-Mensah et al., 2020; Poti et al., 2020). Exon 1 of both genes is 69 bp long coding for 23 amino acid residues. The nucleotide sequences which translate into the histidine-rich residue of *Pf*HRP2 and *Pf*HRP3, which inform their naming, are located within the second exon of each gene and are of low complexity and express mainly tri- or hexapeptide repeats (Poti et al., 2020). Table 2.4 shows frequent *Pfhrp*2 epitope targets of most RDTs.

Table 2.4: Frequent motifs of *Pfhrp2* and MAbs they react with for RDTs diagnostics

Motif	Motif Length	MAbs
AHHAADAHH	9	S2-5, C2-3
AHHASDAHH	9	S2-5
AHHAADAHHA	10	C1-13
AAYAHHAHHAAY	12	Genway
DAHHAHHA	8	3A4/PTL-3
DAHHAHHV	8	1E1-49
DAHHAADAHH	10	2G12-1C12
DAHHVADAHH	10	2G12-1C12
DAHHAADAHHA	11	N7
HATDAHHAAA	10	A6-4
HATDAHHAAD	10	A6-4
TDAHHAADAHHAADA	15	TC-10
YAHHAHHA	8	1E1-49, PTL-3

(Lee et al., 2012)

2.12 Biological Threats to Malaria Diagnosis and Control

Tremendous progress has been made in the fight against malaria (WHO, 2020c). These interventions have led to the eradication of malaria in many countries, including El-Salvador, Belize, Argentina, Paraguay, China, Timor-Leste, Sri Lanka, Maldives, Timor-Leste, Morocco, Cape Verde and Sao Tome. In the last few decades, malaria transmission has been reduced through effective vector control programmes (like ITNs

and IRS), discovery and availability of more effective chemotherapies, and the development and roll-out of a malaria vaccine (WHO, 2020b, 2020c). Notwithstanding these successes, malaria remains one of the most important infectious diseases of man, especially in sub-Saharan Africa and Asia (Gendrot et al., 2019; WHO, 2019b). Much of the challenge to malaria management and control can be blamed on parasite factors such as mutations to important parasite proteins that affect diagnosis and/chemotherapy and the development of resistance in the vector to used insecticides (WHO, 2014, 2015b; AngloGold Ashanti, 2018; Dalrymple et al., 2018).

2.12.1 *Plasmodium falciparum* histidine-rich protein 2 Gene Deletion/Alteration

The *P. falciparum* is the most prevalent human malaria parasite in sub-Saharan Africa (WHO, 2020a). This situation has mainly informed the wide use of *Pf*HRP2-RDTs in many countries, including Ghana. However, *P. falciparum* parasites lacking partial or complete section of the *hrp2* gene confers false *Pf*HRP2-RDT negative results. *Pf*HRP2-RDTs are able to cross-react with *Pf*HRP3 antigen because of their amino acid sequence homology (WHO, 2019a). The occurrence of *Pfhrp*2 and *Pfhrp*3 gene deletions in clinical cases was first reported in Peru in 2010 (Gamboa et al., 2010). Later, similar mutations were reported in parasites isolated in Colombia (2015), Suriname (2015), Brazil and Bolivia (2017), with the prevalence of mutations reported to be between 4 % to 41 % (Murillo et al., 2015; Okoth et al., 2015; Viana et al., 2017). Since then, several countries have reported sporadic and low prevalence of *Pfhrp*2 gene mutations in malaria parasites (Berhane et al., 2018). Between September 2020 and September 2021, *Pfhrp*2/3 gene deletions have been reported in 17 scientific publications from 13 countries (WHO, 2021). Several studies have reported deletion of

Pfhrp2/3 genes in African countries resulting in false RDT negatives across the study areas. These include studies from Angola (Plucinski et al., 2019), Democratic Republic of Congo (Parr et al., 2017), Eritrea (Berhane et al., 2018), Ethiopia (Girma et al., 2019), Ghana (Amoah et al., 2020), Kenya (Beshir et al., 2017; Nderu et al., 2019), Mali (Koita et al., 2012), Mozambique (Gupta et al., 2017), Nigeria (Funwei et al., 2019), Rwanda (Kozycki et al., 2017), Senegal (Wurtz et al., 2013) and Zambia (Kobayashi et al., 2019) as shown in Figure 2.6.

The WHO recently called for urgent measures to address the increasing prevalence of *Pfhrp2* gene deletions in endemic countries especially in the Horn of Africa (WHO, 2021). Target gene deletions are counter-productive to disease diagnoses and eradication and may even erode past gains made in malaria control. *P. falciparum* isolates that lack part or full *Pfhrp*2 gene will not express the HRP2 antigen. In such instances, *Pf*HRP2-RDTs will not be able to detect the marker when used for diagnosis. Although such isolates may have functioning *Pfhrp*3 genes, the natural expression levels of this protein are usually be low and relatively less sensitive to *Pf*HRP2 detecting MAbs (Baker et al., 2005). Such a phenomenon can generate false-negative RDT results, especially in places where there is a higher incidence of *Pfhrp*2-lacking parasites, especially when the settings are lacking other parasitological diagnostics like microscopy.

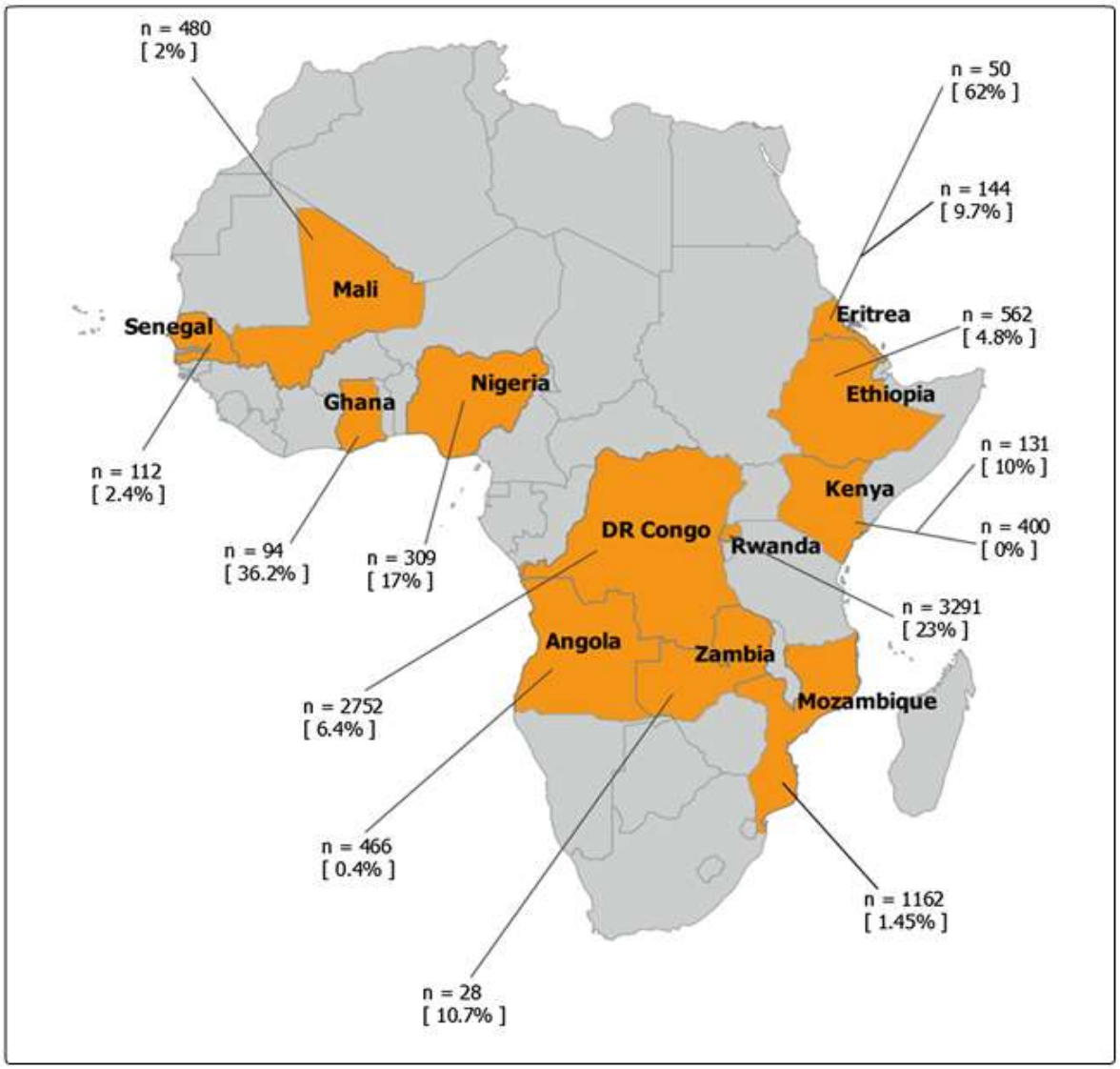


Figure 2.6: Distribution of *Pfhrp2/3* gene deletion in sub-Saharan Africa

Source: Agaba et al. (2019)

2.12.2 Parasites resistance – status of antimalarial drug efficacy

One of the leading threats to malaria control is the parasites' resistance to first-line antimalarial drugs (WHO, 2009). For this reason, the WHO advocates for monitoring antimalarial drug efficacy through therapeutic studies (WHO, 2021). In Ghana, for instance, nationwide studies on the efficacy of chloroquine which was then the first-line

drug against *P. falciparum* malaria, were conducted in 2002 (GHS, 2002). The study reported chloroquine treatment failure to be between 6 % and 25 % and parasite clearance to be as low as 50 % (Koram, 2003; MoH, 2004; Koram et al., 2005). As was the case in several African countries, such revelations triggered a search for alternative treatments for malaria. Thus, Ghana, in particular, changed her anti-malaria drug policy in 2004 to reflect this emerging trend (GHS, 2014b)

Currently artemisinin resistance is being monitored by the WHO using markers to the *PfKelch13* gene, the mutation of which is association with decreased artemisinin sensitivity (Iriart et al., 2020; Srisutham et al., 2020; Al-Awadhi et al., 2021; WHO, 2021). Shreds of evidence of artemisinin resistance and clonal expansion of *PfKelch13* marker mutations have been reported in Asia and in some countries in Africa, including Rwanda and Uganda (Phyo and Nosten, 2018; Fidock and Rosenthal, 2021; Ghanchi et al., 2021; Uwimana et al., 2021; Van Loon et al., 2022). However, treatment failure rates in these countries fall below the threshold of classification as drug resistance (WHO, 2020a). Therapeutic efficacy studies on ACTs conducted using WHO standard protocol showed high efficacy, and thus, these drug combinations have been the first-line treatments of *P. falciparum* malaria in sub-Saharan Africa from 2015 to date (WHO, 2021). However, a candidate marker of ACT partial resistance, also called R622I, has been identified in increasing rate in malaria vectors particularly in Eritrea, Ethiopia, Somalia and Sudan (WHO, 2020c). The real impact of this mutation on parasite resistance and drug efficacy is yet to be fully evaluated (WHO, 2020c).

2.12.3 Vector Resistance to insecticides

The most successful malaria preventive measures have been IRS and the ITNs (WHO, 2010b; Kim et al., 2012). An important component of these preventive methods is insecticides such as pyrethroids, organochlorines, carbonates and organophosphates (Killeen et al., 2017; WHO, 2021). Insecticides used to control mosquitoes must be very safe, friendly to the environment and effective (WHO, 2010b). Insecticide resistance by mosquitoes stands as a threat to global malaria control and eradication programmes and calls for urgent measures to slow the spread of established resistance mechanisms and prevent the development of new ones (WHO, 2020b).

Resistance to pyrethroids, the primary insecticide class used in ITNs for decades, has been recorded in not less than one malaria vector in 68 % of areas where data is available. This is thought to be associated with the usage of the chemical as a single line insecticide (WHO, 2010b, 2021). Pyrethroid-piperonyl butoxide (PBO) combination treatments have been reported to have the ability to restore mosquito susceptibility to pyrethroids and should be promoted (WHO, 2019b). Mosquito resistance to pyrethroids was first published in 1996 and seems to be on the rise (Pasteur and Raymond, 1996; Lemine et al., 2018;). Most IRS schemes employ the use of organophosphate and neonicotinoid insecticides (AngloGold Ashanti, 2018). Out of 88 countries that reported insecticide resistance from 2010-2020, 78 confirmed vectors resistances to at least one insecticide from one mosquito sampling site but 29 confirmed resistances to four (4) insecticides classes, namely, pyrethroids, organophospahtes, carbamates and organochlorines. Due to the lack of data about mosquito resistance and/or susceptibility to ITN and IRS insecticides from many countries, there exists a challenge to the

development of effective strategies to prevent and control resistance to effective insecticides used in malaria control (Suuron et al., 2020).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Sites/Area

Four healthcare facilities in three districts of northern Ghana (Figure 3.1), namely, the Tamale Teaching Hospital, Tamale Central Hospital and Yendi Municipal Hospital in the Northern Region, and Damango Municipal Hospital in the Savannah Region, were selected as study sites. The health facilities were selected because they reported the top four highest OPD attendants in the former Northern region of Ghana. The Northern and Savannah Regions are characterized by Guinea savannah vegetation and have a population of around 2, 964, 205 inhabitants (GSS, 2021).

The Tamale Teaching Hospital, a tertiary referral hospital and Tamale Central hospitals, a secondary healthcare institution which offers general services, are two of the few healthcare facilities in the Tamale Metropolis that provides healthcare services to inhabitants of Tamale and its environs. Tamale metropolis is one of the 14 districts of the Northern Region and lies between longitudes 0° 36 and 0° 57 West and latitude 9° 16 and 9° 34 North, with about 115 communities. The district capital, Tamale, couples as the regional capital of the Northern Region (GSS, 2014a).

The Yendi Municipal Hospital is the only secondary healthcare facility in the municipal. Yendi municipal can be found in the eastern corridor of the Northern Region and shares boundaries with about six districts. The district capital, Yendi, is ~90 km from Tamale.

The Damango Municipal Hospital is situated in Damango, the capital town of the Savannah Region, one of the new regions carved out of the Northern Region on 12th

February, 2019. Damango couples as the district capital of West Gonja Municipality (GSS, 2014b). The hospital is the highest healthcare facility in the municipality and is stated to provide healthcare to about 63,449 people (GSS, 2021).

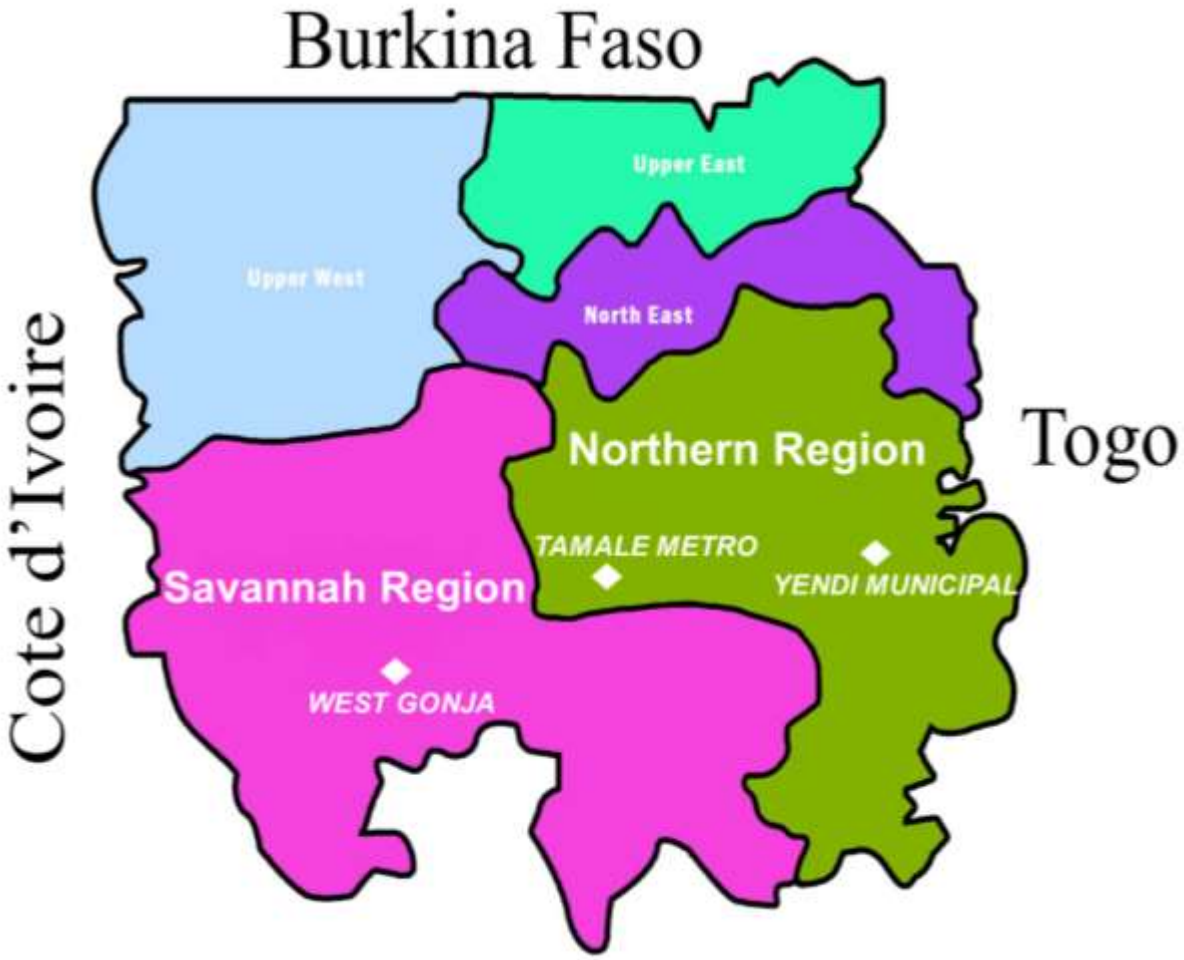


Figure 3.1: A map of northern Ghana showing the districts in which the study facilities are located

Adapted:https://citinewsroom.com/wp-content/uploads/2019/02/16-regions-of-Ghana.jpeg

Date accessed: 16.11.2021

3.2 Study Design and Inclusion/Exclusion Criteria

A cross-sectional serological and molecular survey was carried out between December 2020 and May 2021 for the study on blood samples of persons presenting with fever (as assessed by a clinician) and seeking healthcare in the study facilities after informed consent was acquired. Blood samples collected from study participants were tested by microscopy and confirmed to be positive for *Plasmodium* species. Patients who presented with fever but tested negative for microscopy were excluded from the study.

3.3 Sample Collection and Microscopy

A total of 267 participants were purposively recruited across the out-patient department (OPD) of the four study sites. About 5 ml venous blood samples were collected by venipuncture in heparin-coated vials in the laboratories of the health facilities. From the vial, thick and thin blood smears were prepared with 2 µl and 6 µl of blood samples, respectively for quantification and microscopic detection of *Plasmodium* parasites using the Giemsa stain method in the laboratories of the respective health facilities.

Thick blood smears were prepared as described by Bailey et al. (2013) with few modifications. Briefly, 6 µl of the venous blood sample was dropped on a glass slide and allowed to air-dry for 60 min at room temperature. The dried smear was fixed in acetone for 10 min and then stained in 10 % Giemsa solution (Sigma-Aldrich, USA) for 15 min. Excess stain from both sides of the slide was washed off with a strong jet of deionized water and air-dried. The stained smear was analyzed under a light microscope using the X100 objective with oil immersion.

The thin blood smears were prepared by dropping 2 µl of fresh blood sample on a glass slide. Aided by a second glass slide, the drop was immediately spread smoothly and gently over the surface of the slide and allowed to air-dry for 10 min. The dried blood was fixed for 10 s in 100 % methanol and stained in 10 % Giemsa solution for 15 min. The stained slide was visualized under a light microscope after washing off the excess stain. Slide analyses were performed by two microscopists with a minimum of five working years experience from each healthcare facility following standard procedure (Bailey et al., 2013). Each microscopist examined every sample independently. A sample was declared positive for *Plasmodium* infection only when both microscopists recorded positive for both thin and thick smears of a sample. In the case where one microscopist records positive for a sample and the other records negative, a third microscopist in the facility was invited to analyze the sample. Samples that tested positive for malaria, thus quantification of malaria parasites by microscopy were used to prepare three dried blood spots on Whatman 903™ filter paper (GE Healthcare Bio-Sciences Corp.), labelled and stored in air-tight plastic zip lock bags. Dried blood spot samples were transported to the Spanish Laboratory Complex, Nyankpala Campus, University for Development Studies for molecular analyses.

3.4 Malaria Rapid Diagnostic Test

Blood samples that tested positive for malaria by microscopy were used for malaria RDT using CareStart™ Malaria *Pf*HRP2-RDT kit (AccessBio Inc, Somerset, NJ, USA) following the manufacturer's protocol for rapid diagnosis. Briefly, about 6 – 10 µl of the venous blood was dispensed into the sample well of the RDT cassette using the capillary pipette. Afterwards, two drops (~60 µl) of buffer solution were added into the

analyte "A" well on the disk and result was read in 20 min. Test interpretation was done following the manufacturer's instructions. A positive result was indicated by the presence of two bands ("C" and "T" indicators) in the control area of the strip whereas negative results were indicated by the presence of only one band next to the "C" indicator on the result window.

3.5 Protozoan DNA Extraction

A modified Tris-EDTA buffer extraction method (Bereczky et al., 2005) was used to extract protozoan genomic DNA (gDNA) from dried blood spots on filter paper. Briefly, punch of approximately 3.75 mm^2 was made a dried blood spot on a filter paper and placed in a 0.2 ml tube containing 98 µl of TE buffer, pH 8.0 [10 mM Tris (Tris-base plus Tris-HCl) and 0.1 mM EDTA] and then incubated at 50 °C for 30 min in peqSTAR 96X Universal thermal cycler (peQlab, VWR). After incubation, the punched dried blood spot filter paper was pressed gently to the bottom of the tube several times using a sterile pipette tip and heated at 97 °C for 15 min in peqSTAR 96X Universal thermal cycler (peQlab, VWR) to elute the DNA. The lysates were centrifuged for 10 s at 2,500 g in a Centro-8 Centrifuge (J. P. Selecta) to separate cell organelles. The suspension (DNA extract) was used immediately for PCR or stored at -20 °C for use later.

3.6 Polymerase Chain Reaction Amplification of the 18S rRNA of *Plasmodium falciparum*

A semi-nested PCR assay that amplified the 18S rRNA gene of *P. falciparum* was performed using peqSTAR 96X Universal thermal cycler (peQlab, VWR). The primary

(1°) PCR consisted of 5 µM each of primer (forward - 5' – AGT GTG TAT CCA ATC GAG TTT C – 3' and Reverse - 5' – GAC GGT ATC TGA TCG TCT TC – 3') (Rubio et al., 1999), 20 mM Tris-HCl (pH 8.9 at 25 °C), 1.8 mM $MgCl_2$, 22 mM NH_4Cl, 0.2 mM dNTPs, 5 % glycerol, 0.06 % IGEPAL® CA-630, 0.05 % Tween- 20, xylene Cyanol FF, Tartrazine, 0.125U One *Taq*® DNA polymerase (New England Biolabs® Inc) and 1 µl DNA extract as template in a 10 µl final reaction volume. The reaction was cycled using the thermal conditions shown in Figure 3.2.

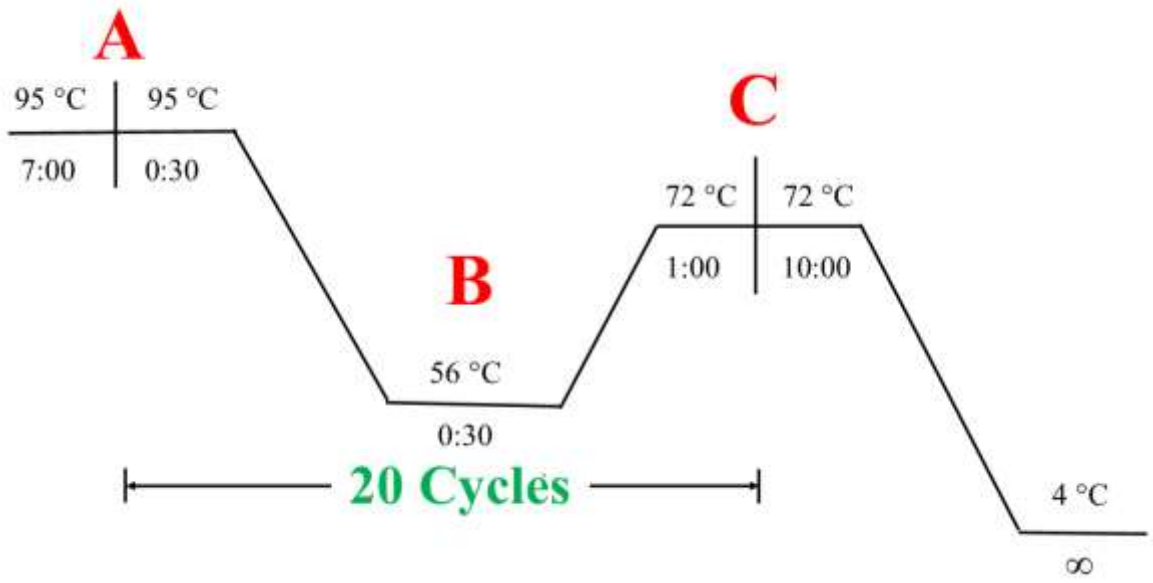


Figure 3.2: Primary PCR Cycling Conditions for amplification of the 18S rRNA gene of *P. falciparum*

A – Denaturation Step; B – Annealing step; C – Elongation/Extension step

The semi-nested PCR consisted of 5 µM of each primer (forward - 5' – AGT GTG TAT CCA ATC GAG TTT C – 3' and reverse - 5' – AGT TCC CCT AGA ATA GTT ACA – 3'), 20 mM Tris-HCl (pH 8.9 at 25 °C), 1.8 mM $MgCl_2$, 22 mM NH_4Cl, 0.2 mM dNTPs, 5 % glycerol, 0.06 % IGEPAL® CA-630, 0.05 % Tween- 20, xylene Cyanol FF,

Tartrazine, 0.625 U One *Taq*® DNA polymerase (New England Biolabs® Inc) and 4 µl of primary PCR amplicons as template in a 50 µl final reaction volume. The semi-nested PCR was cycled using the thermal conditions illustrated in Figure 3.3. A negative control of nuclease-free water and a positive control composed of *P. falciparum* 3D7 gDNA that was acquired from WACCBIP (West African Centre for Cell Biology of Infectious Pathogens) in the University of Ghana, were included in all PCRs to check the performance of all reactions.

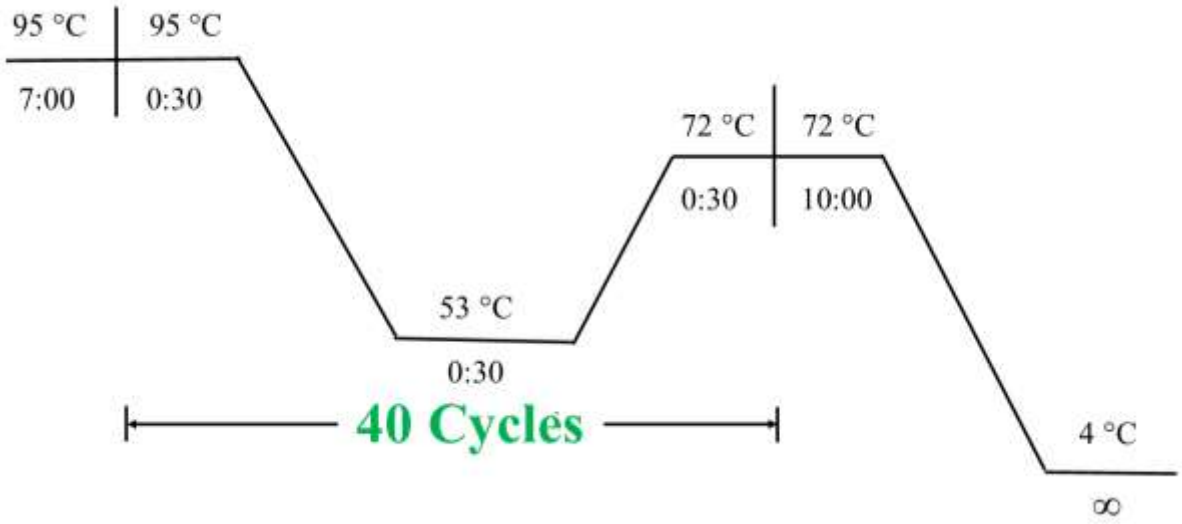


Figure 3.3: Semi-nested PCR Cycling Conditions for the amplification of the 18S rRNA gene of *P. falciparum*

3.7 *Plasmodium falciparum* histidine-rich protein 2 and 3 Genotyping

A nested PCR was performed to detect the antigenic genes *Pfhrp*2/3 that are targeted by the CareStart™ Malaria *Pf*HRP2-RDT kit (AccessBio Inc, Somerset, NJ, USA) used in this study using the primers (Table 3.1) designed by Abdallah et al. (2015).

Primary (1°) PCR for both markers were run in 10 µl reaction volume constituting of 5 µM of each primer, 20 mM Tris-HCl (pH 8.9 at 25 °C), 1.8 mM $MgCl_2$, 22 mM NH_4Cl, 0.2 mM dNTPs, 5 % glycerol, 0.06 % IGEPAL® CA-630, 0.05 % Tween- 20, xylene Cyanol FF, Tartrazine, 0.125U One *Taq*® DNA polymerase (New England Biolabs® Inc) and 1 µl DNA extract. The reaction was cycled using the conditions: initial denaturation at 95 °C for 10 min, 20 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s (*Pfhrp*2) and 55 °C for 30 s (*Pfhrp*3) and elongation at 72 °C for 60 s, and extension at 72 °C for 10 min.

In the nested PCR, 4 µl of 1° PCR amplicon was used as template in a 50 µl reaction volume that consisted of same constituents as in the 1° PCR but with 0.625 U One *Taq*® DNA polymerase (New England Biolabs® Inc). The nested reaction was run using the cycling conditions as follows: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s (*Pfhrp*2) and 60 °C for 30 s (*Pfhrp*3) and elongation at 72 °C for 30 s, and extension at 72 °C for 10 min. A negative control composed of nuclease-free water and a positive control composed of the *P. falciparum* 3D7 gDNA (see section 3.5) were included in all PCRs.

All PCRs were run in the peqSTAR 96X Universal thermal cycler (peQlab, VWR) (Appendix I) in the Microbiology/Biotechnology laboratory of the Spanish Laboratory Complex, University for Development Studies, Nyankpala Campus. Also, all reactions that showed no amplification were re-run one more time for certainty.

Table 3.1: Primers used in PCR amplification of Exon 1−2 of *Pfhrp*2 and 3 genes and their expected fragment sizes (Abdallah et al., 2015)

Target Marker	PCR	Primer Pairs (5' – 3')	Expected Amplicon Size
*Pfhrp*2 Exon 1-2	1° PCR	F: GGT TTC CTT CTC AAA AAA TAA AG	228 bp
		R: TCT ACA TGT GCT TGA GTT TCG	
	Nested PCR	F: GTA TTA TCC GCT GCC GTT TTT GCC	
		R: CTA CAC AAG TTA TTA TTA AA TGC GGA A	
*Pfhrp*3 Exon 1-2	1° PCR	F: GGT TTC CTT CTC AAA AAA TAA AA	225 bp
		R: CCT GCA TGT GCT TGA CTT TA	
	Nested PCR	F: ATA TTA TCG CTG CCG TTT TTG CT	
		R: CTA AAC AAG TTA TTG TTA AAT TCG GAG	

Exon 1−2: spanning the region of exon 1 through intron to the beginning of Exon 2 but no exon 2; F: Forward Primer; R: Reverse Primer

3.8 Gel Electrophoresis

All PCR products were resolved on 2.0 % (w/v) agarose gel (peqGOLD, VWR International). Agarose gel was prepared by dissolving 1 g of agarose granules in 50 ml 1x Tris-Boric acid-Ethylenediaminetetraacetic acid (TBE) buffer (pH 8.3). The mixture was microwaved for 80 s for complete dissolution and stained with ethidium bromide, swirled gently and cast into prepared gel tray and allowed to solidify for ~ 30 min. Seven microlitres of PCR amplicons were loaded into wells of gel and run at 80 V for 40 min. Size of fragments were estimated by comparing with NEB's Quick-Load® Purple 100 bp DNA Ladder [2.5 % Ficoll®-400, 10 mM EDTA, 3.3 mM Tris-HCl, 0.001 % Dye 2,

0.02 % Dye 1, pH 8 @ 25 °C]. Gels were visualized under 21 x 26 cm UV Transilluminator and images captured with adjoining MicroDOC Gel Documentation System (Cleaver Scientific Ltd., UK) (Appendix II).

3.9 DNA Sequencing

In all, 40 PCR amplicons of the 18S rRNA (n = 4) and exon 1–2 of *Pfhrp2* (n = 19) and *Pfhrp3* (n = 17) genes of *P. falciparum* were sequenced (Inqaba Biotechnical Industries (pty) Ltd., Pretoria, South Africa). Isolates were sequenced using the respective reverse primers as in the nested PCR.

3.10 Case definitions

True-positive (TP) RDT results were defined as positive RDT results of samples that were also positive for *P. falciparum* by PCR. True-negative (TN) RDT results were defined as negative RDT results of samples that were also negative by PCR. False-positive (FP) RDT results were defined as positive RDT results of samples that were negative by PCR and false-negative (FN) RDT results were defined as negative RDT results of samples that were positive for *P. falciparum* by PCR.

3.11 Statistical and Molecular Analyses

Demographic and clinical data of participants, including microscopy, RDT and PCR-based assays statuses were entered into Microsoft Excel suite 2019 (Microsoft, USA) for descriptive statistical analysis. Stata statistical software v. 14.2 (StataCorp LLC, Texas, USA) was used to calculate the mean and to determine the chi-square (π^2) values of positivity rates among diagnostic methods. DNA sequence data were viewed and

edited using the GENtle software (http://gentle.magnusmanske.de/) version 1.9.4 (Manske, 2006).

Gene model description of PF3D7_0831800.1 (*Pfhrp*2) and PF3D7_1372200 (*Pfhrp2*) were inferred from PlasmoDB Informatics Resources (https://plasmodb.org/). The nucleotide Basic Local Alignment Search Tool (BLASTn) algorithm of the National Centre for Biotechnology Information (NCBI) was used to confirm amplified 18S rRNA marker of *P. falciparum* and histidine-rich protein 2 and 3 and denote its similarity to already deposited sequences in GenBank.

The Molecular Evolutionary Genetics Analysis X (MEGA X) software (https://www.megasoftware.net/) version 10.2.6 (Kumar et al., 2018) was used for in-silico translation of nucleotide sequences (exons of *Pfhrp*2 and 3) to their corresponding amino acids with the correct open reading frame. MEGA X was also used in drawing the phylogenetic relationship between isolates of the present study and WHO approved strains and other *P. falciparum* sequences. DnaSP (http://www.ub.edu/dnasp/) version 6.12.03 (Rozas et al., 2017) PopART and TCS (Clement et al., 2002; Leigh and Bryant, 2015) software was used for nucleotide variability analyses of partial intron sequences of amplified *Pfhrp*2 and 3 genes. All sequence alignments were done by CLUSTAL Omega on the EMBL-EBI online portal (https://www.ebi.ac.uk/Tools/msa/clustalo/).

3.12 Ethics and Consent

Ethical and administrative approval was obtained from the School of Medicine and Health Sciences and School of Allied Health Sciences Joint Institutional Review Board (SMHS-SAHS JIRB) of the University for Development Studies (Approval Certificate

ID: SMSAHS/JIRB/0010) (Appendix III). Written informed consent was obtained from all adults who participated in the study. On behalf of persons below 18 years, written informed consent was obtained from caregivers.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic Characteristics of Study Participants

Across the four study centres, 267 participants were recruited (Table 4.1). Of these 267 study participants, 158 (59.20 %) were females and 40.8 % (109/267) were males. The age distribution of participants was from 3 weeks to 88 years, with the mean age of participants being 19.3 years (SD±17.2). However, 32.21 % of participants were children who were 5 years or below.

Table 4.1: Sex and age distribution of study participants from health facilities

Age Groups	Health Facility/Sex Distribution							
	YMH		TTH		TCH		DMH	
	Female	Male	Female	Male	Female	Male	Female	Male
>5 years	8	7	10	13	9	18	3	3
5–14 years	9	10	4	8	6	9	3	2
15–35 years	10	10	24	5	31	13	14	5
36–59 years	2	0	7	7	8	5	4	1
≥60 years	0	0	5	1	1	1	0	1

TTH: Tamale Teaching Hospital, TCH: Tamale Central Hospital, DMH: Damango Municipal Hospital, YMH: Yendi Municipal Hospital

4.2 Identification of Malaria Parasites

4.2.1 Microscopy confirmation of *Plasmodium* infection

All 267 blood samples across the four study sites were confirmed positive for malaria by identification of malaria-causing *Plasmodium* spp. by microscopy (Table 4.2).

Table 4.2: *Plasmodium* spp. identified by the Giemsa-stained microscopy technique

***Plasmodium* spp.**	**No. of Isolates**	**Percentage (%)**
P. falciparum (*Pf*)	264	98.88
P. malariae (*Pm*)	2	0.75
Mixed (*Pf* and *Pm*)	1	0.37

Results of microscopy examination of thin blood smear (Table 4.3) revealed that the total parasitemia of the study samples was between 40 and 150,000 parasites/µl and all participants received malaria treatment after microscopy confirmation of *Plasmodium* infection.

Table 4.3: Parasitic loads of study samples across study sites

STUDY SITE (ŋ)	**PARASITAEMIA (p/µl)**				
	≤200	***201-999***	***1000-4999***	***5000-9999***	***≥10,000***
Tamale Teaching Hospital (ŋ=84)	19	14	17	7	27
Tamale Central Hospital (ŋ=101)	39	23	18	8	13
Damango Municipal Hospital (ŋ=36)	ND	ND	ND	ND	ND
Yendi Municipal Hospital (ŋ=46)	0	9	11	7	19

ND – Not determined

4.2.1 Rapid Diagnostic Tests (RDTs)

Overall, the RDT kits detected *P. falciparum* infection in 59.2 % (158/267) of the samples (Table 4.4). Facility-specific positivity numbers stood at 49/84 (58.3 %), 49/101 (48.5 %), 32/36 (88.9 %) and 28/46 (60.9 %) for TTH, TCH, DMH and YMH hospitals, respectively.

Table 4.4: Positivity rate of Rapid Diagnostics Tests across study facilities

RDT Status	Health Facility				Total
	YMH	TTH	TCH	DMH	n (%)
Negative	18	35	52	4	109 (40.8)
Positive	28	49	49	32	158 (59.2)

4.2.2 PCR Detection of *Plasmodium falciparum*

The semi-nested PCR assay generated an amplicon size of approximately 395 bp (Figure 4.1) of the target gene for each positive sample.

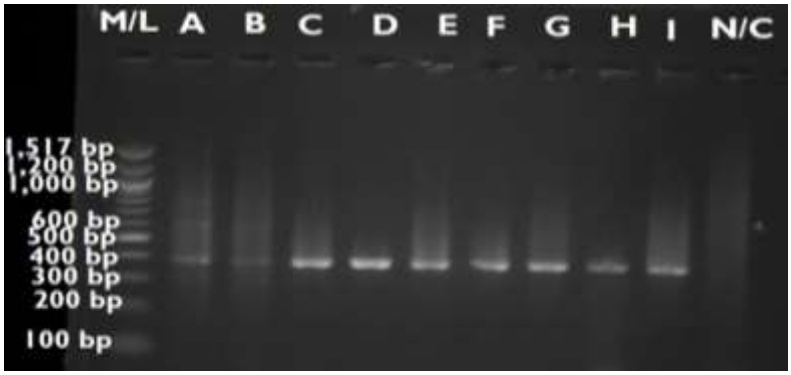


Figure 4.1: Results of *P. falciparum* 18S rRNA PCR ran on 2 %, (w/v) agarose gel showing the approx. 395 bp size.

M/L: Molecular Ladder (Quick-Load® Purple 1 kb DNA Ladder); Lane A-I: *P. falciparum* positive samples showing amplification; N/C: Negative Control.

In total, 160/267 (59.92 %) out of the total of 267 samples tested were positive for *P. falciparum* by semi-nested PCR amplifications (Appendix IV). Across the study sites, *P. falciparum* genetic material was detected in 47/84 (56.00 %), 61/101 (60.40 %), 27/36 (75.00 %) and 25/46 (54.30 %) for TTH, TCH, DMH and YMH facilities, respectively. Comparison of the positivity rates of RDT and PCR in the diagnosis of *P. falciparum* are presented in Figure 4.2.

Study facility had statistical significance to outcomes of diagnoses by RDT (π^2=17.987, p-value=0.0001) but not significant to PCR-based assay (π^2=4.564, p-value=0.207). However, outcomes of the two diagnostic methods were independently exclusive (π^2=59.341, p-value=0.0001).

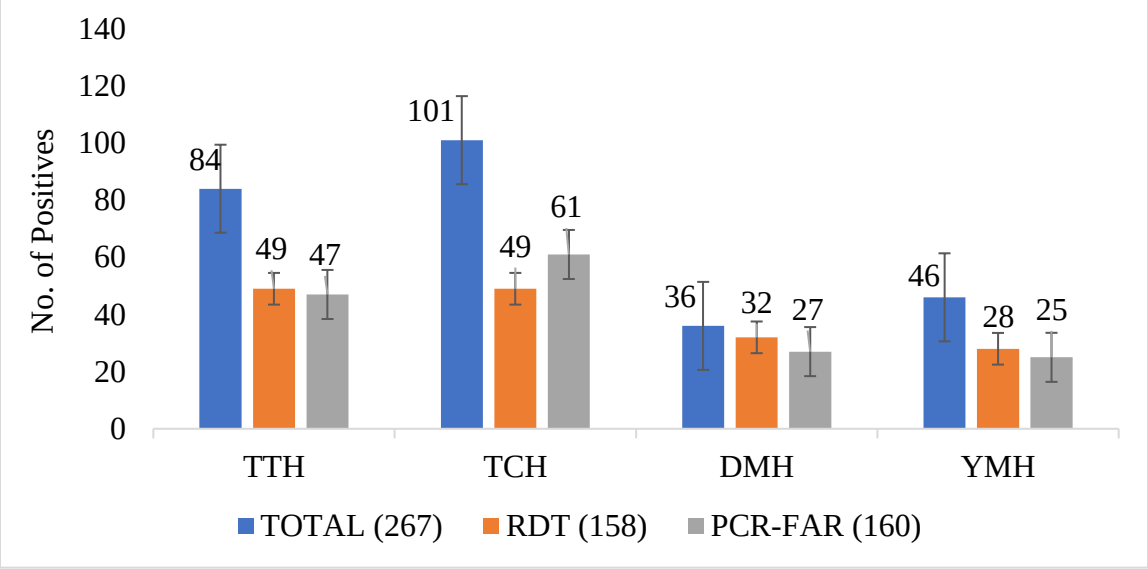


Figure 4.2: PCR-*Plasmodium falciparum* and *Pf*HRP2-RDT positivity rates across the study sites. (Error bars indicate the standard variability in reported data)

4.3 False Positive and False Negative RDT Results

From the present study, PCR assay revealed 107 samples out of the total of 267 samples to be negative for *P. falciparum*. Out of the 107 parasite negative results by PCR, 33 were positive for *P. falciparum* by RDT, indicating a 30.8 % false positive RDT rate. Among the different study facilities, TTH showed RDT false positive rate of 24.3 % (9/37), while TCH, DMH and YMH showed RDT false positive rates to be 22.5 % (9/40), 77.7 % (7/9) and 38.1 % (8/21), respectively

Once PCR had identified the presence of *P. falciparum* in blood sample, it was expected that the *Pf*HRP2-RDT would give a positive result as well. From the present study, PCR assay revealed 160 samples out of the total of 267 samples to be positive for *P. falciparum*. Out of the 160 parasite positive results by PCR, 35 were negative for *P. falciparum* by RDT, indicating a 21.9 % false negative RDT rate. Among the different study facilities, TTH showed RDT false negative rate of 14.9 % (7/47), while TCH, DMH and YMH showed RDT false negative rates of 34.4 % (21/61), 7.4 % (2/27) and 20.0 % (5/25), respectively

4.4 *Plasmodium falciparum* Histidine-Rich Protein 2 and 3 (*Pfhrp2* and *3*) Genes Genotyping

The amplicon sizes for the target sequences of *Pfhrp2* and *Pfhrp3* were approximately 228 bp (Figure 4.3) and 225 bp (Figure 4.4), respectively.

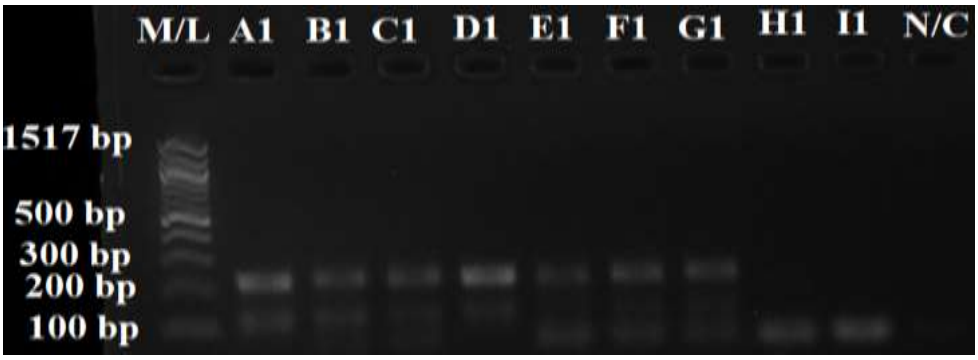


Figure 4.3: Results of PCR targeting *Pfhrp*2 gene segment spanning exon 1−2 ran on 2 % (w/v) agarose gel.

M/L: Molecular Ladder (Quick-Load® Purple 1 kb DNA Ladder); Lanes A1-I1: presence/absence of *Pfhrp2* gene in blood samples; N/C: Negative Control (No DNA Template)

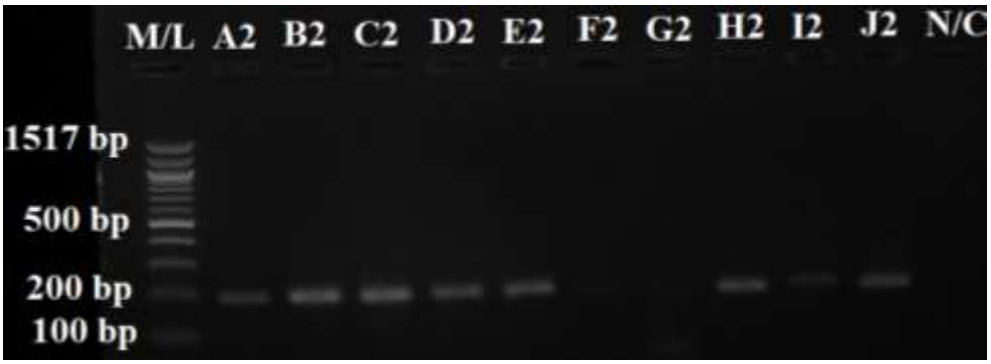


Figure 4.4: Results of PCR targeting *Pfhrp*3 gene segment spanning exon 1−2 ran on 2 % (w/v) agarose gel.

M/L: Molecular Ladder (Quick-Load® Purple 1 kb DNA Ladder); Lanes A2-J2: presence/absence of *Pfhrp*3 gene in blood samples; N/C: Negative Control (No DNA Template)

All 160 samples that were confirmed by PCR to be positive with *P. falciparum* were subjected to *Pfhrp*2 and 3 screening (Appendices V and VI). Out of these, 19 representing 11.9 % and 22 representing 13.8 % of the samples lack or had mutation of the exon1-2 fragment of *Pfhrp*2 and *Pfhrp*3 genes, respectively (Table 4.4). The phenomenon of both genes was observed in 17 samples representing 10.6 % whereas, 24 samples lack or had both or one of the antigenic genes, mutated. Out of this number, 20 tested negatives for RDT which confirms false RDT negative by absence or mutation of the antigenic protein(s).

Table 4.5: Frequency of *Pfhrp*2 and 3 presence and absence in study samples

Study Site	***Pfhrp*2-/*Pfhrp*3-**	***Pfhrp*2-/*Pfhrp*3+**	***Pfhrp*2+/*Pfhrp*3-**	***Pfhrp*2+/*Pfhrp*3+**	**Total**
Tamale Teaching Hospital	7	1	0	39	47
Tamale Central Hospital	5	0	2	54	61
Damango Municipal Hospital	1	1	0	25	27
Yendi Municipal Hospital	4	0	3	18	25
Total	**17**	**2**	**5**	**136**	**160**

*Pfhrp*2- means no amplification observed after *Pfhrp*2 Exon 1–2 PCR; *Pfhrp*2+ means ~228 bp amplicon observed after *Pfhrp*2 Exon 1–2 PCR; *Pfhrp*3- means no amplification observed after *Pfhrp*3 Exon 1-2 PCR; *Pfhrp*3+ means ~225 bp amplicon observed after *Pfhrp*3 Exon 1-2 PCR

4.5 Genetic Microvariations of *Plasmodium falciparum* histidine-rich protein 2 (*Pfhrp*2)

Sixteen out of 19 amplicons of the *Pfhrp*2 (Exon 1–2) gene were sequenced, producing an average fragment length of 184 bp. All the 16 sequences were 99.17 – 99.77 % identical to *P. falciparum* histidine-rich protein 2 gene sequences already deposited in GenBank including that of the *P. falciparum* 3D7. DNA sequences matched Exon–1 (position 1714 – 1758) and the ensuing intron (position 1759 – 1904) of the *P. falciparum* 3D7 reference strain using the Gene Identification Number Pf3D7_0831800 from Plasmodb as a reference sequence.

4.5.1 Variation and In-silico Analyses of Exon1

The multiple sequence alignment between 45-bp fragments of exon1 of *Pfhrp*2 gene of each of the 16 sequenced data from the present study with that of the reference 3D7 strain (Pf3D7_0831800) revealed that 3/16 (18.75 %) sequences were conserved to the reference strain. Also, point mutations were observed in several samples across the 19 variable sites in at least one sample of the present study (Figure 4.5). Nucleotide diversity (π) and haplotype (gene) diversity (Hd) among 16 *Pfhrp2* Exon1 sequences were 0.10889 and 0.925, respectively.

Figure 4.5: Alignment of sequenced data of exon1 of the *Pfhrp*2 gene with *P. falciparum* 3D7 reference strain; * means 100 % identity at the particular nucleotide site.

In-silico translation of 16 *Pfhrp2*-Exon1 sequences of the present study generated 15 amino acid (AA) residues except for one sequence (DM036) that generated 14 AAs (Figure 4.6). Unlike their DNA sequences, 8/16 (50 %) AA residues matched 3D7 reference strain for all sites.

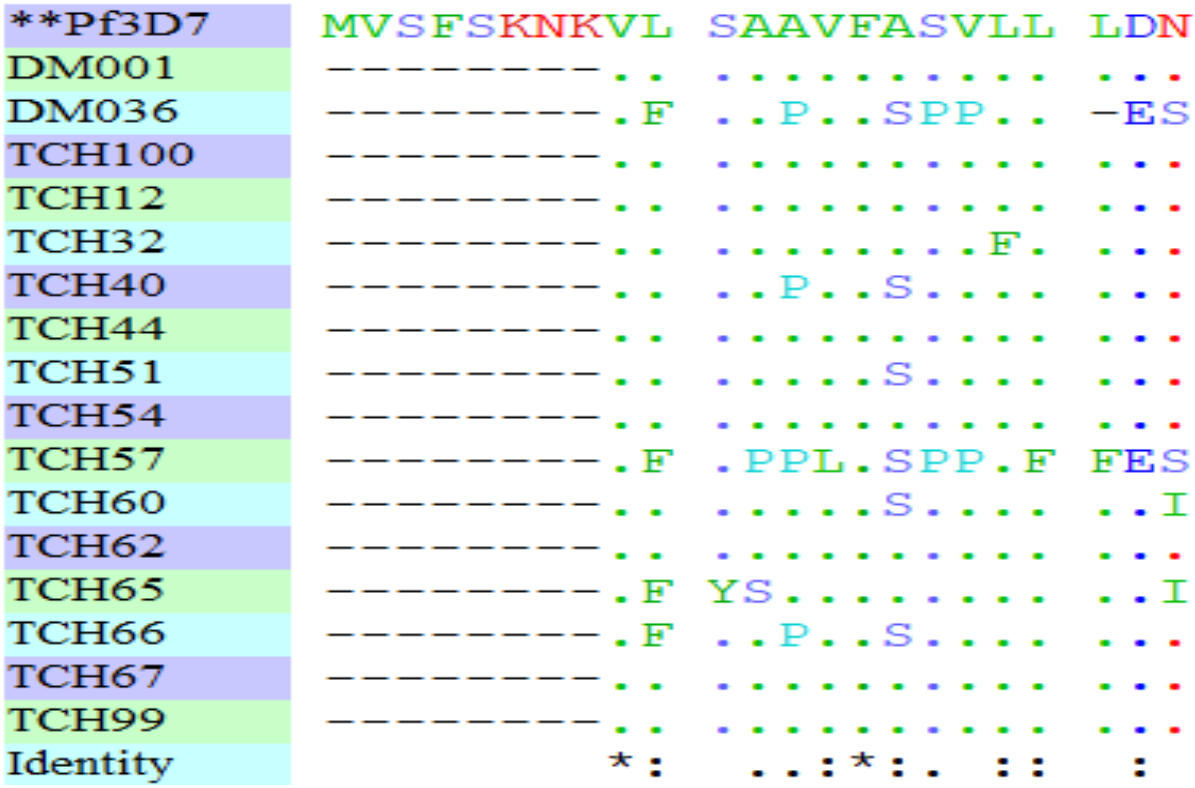


Figure 4.6: Multiple sequence alignment of translated exon 1 residues of *Pfhrp*2 from 16 samples of the present study to *P. falciparum* 3D7 reference strain. * means 100 % identity at the particular residue site

4.5.2 Haplotype Analysis of *Plasmodium falciparum* histidine-rich protein 2–Intron (145 bp)

The intron sequences of all 16 isolates were spliced, aligned and used for haplotype analyses. In all, 13 haplotypes were identified, of which haplotypes H1 and H2 had frequencies of three and two isolates, respectively, whereas the remaining 11 haplotypes occurred only once. Gene diversity occurrence was about 96.7 % across all aligned variable sites.

From Figure 4.7, a dispersive distribution fashion with 2 – 12 mutational steps between neighbouring haplotypes were observed. The network also revealed seven hypothetical haplotypes that were not seen among the isolates analysed in the present study but aided the formation of network loops. The haplotype analysis revealed 25 variable sites (S) with 0.05273 nucleotide diversity.

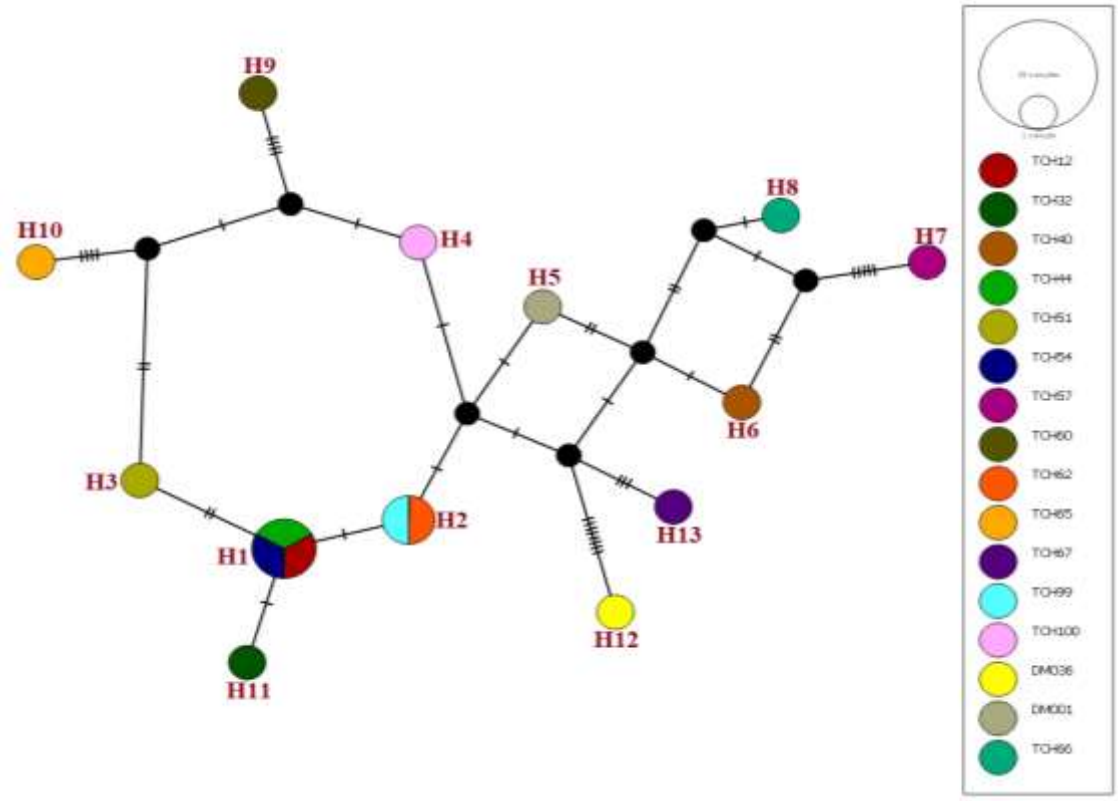


Figure 4.7: Haplotype network of *Pfhrp*2 (Introns) sequences indicating variabilities between isolates of the present study.

Haplotype frequency is proportional to vertex size and mutational steps are shown by number of hatch marks between vertices. The black dots/circles are hypothetical haplotypes not seen among isolates of the present study.

4.5.3 Phylogenetic Analysis of *Plasmodium falciparum* histidine-rich protein 2 (*Pfhrp*2) Sequences

All the 16 sequenced data of the partial fragment (184 bp) of *Pfhrp*2 gene of the present study and that from previous GenBank and Plasmodb deposits were used for phylogenetic analysis. The unrooted tree showed 0.050 (5 %) number of substitutions per aligned sites, thus, five substitutions per 100 bp. The isolates of the present study clade the basal part of the tree indicating that they are earlier ancestors to all other sequences except for PfNF166 and X69922 (Figure 4.8). However, the monophyletic clade, TCH66, TCH57 and DM36 are one of the first ancestors and higher sequence divergence to all sequences under consideration. Except for TCH44 and TCH54, all the sequences of the present study shared a paraphyletic relation with Pf3D7 and other diagnostic reference strains.

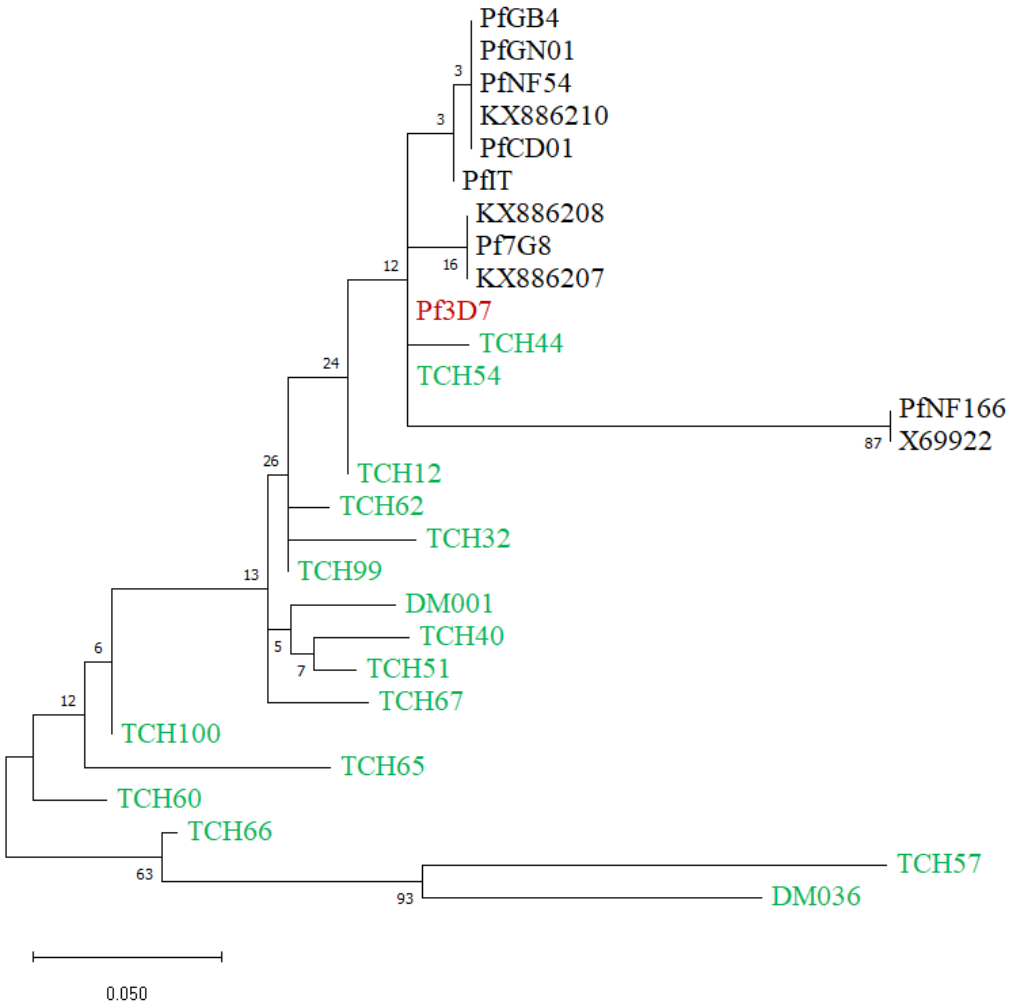


Figure 4.8: A Maximum Likelihood (ML) Phylogenetic tree of *P. falciparum* isolates based on the histidine-rich protein 2 (Exon 1–2) sequences of the present study analyzed with reference deposits.

Sequences of the present study are shown in green font and ML tree was inferred on the Tamura-Nei Model with 1000 bootstrap replications.

4.6 Genetic Microvariations of *Plasmodium falciparum* histidine-rich protein 3 (*Pfhrp*3) Gene

Sequencing of the 16 amplicons of the *Pfhrp3* gene of *P. falciparum* from the samples of the present study yielded a 153 bp cleaned length for molecular analyses. The sequence spanned between 39 and 45 bp of exon 1 of the gene and around 108 bp of the ensuing intron.

4.6.1 Variation and In-silico Analyses of Exon1 of the *Pfhrp*3 Gene

The multiple sequence alignment between *Pfhrp3*-exon1 sequences of the present study showed no conservation across all genetic sites between themselves. This makes the gene diversity between the 16 isolates absolute (1.000) with 20 polymorphic (segregating) sites and 0.14649 nucleotide diversity (π). However, 18 nucleotide sites showed a point resemblance to the reference *P. falciparum* 3D7 strain sequence as TCH66 showed conservation with the 3D7 reference strain (Figure 4.9).

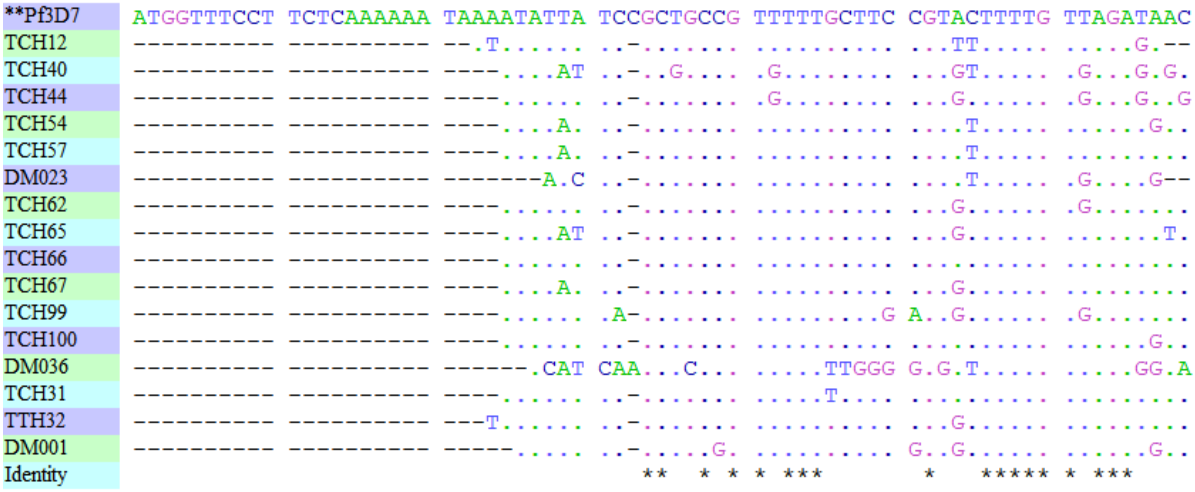


Figure 4.9: Multiple sequence alignment of *Pfhrp*3–exon1 sequences of present study to *P. falciparum* 3D7 reference strain.

* means 100 % identity at the particular nucleotide site.

Comparing the *Pfhrp*3–exon1 amino acid sequence of the present study to *P. falciparum* 3D7 reference strain showed that none of the 16 isolates had 100 % array of amino acid residues similar to the reference. The similarity was, however, seen from residue 12 to 18 of 14 isolates of the present study to the reference strain (Figure 4.10). In addition, 10 isolates of the present study had isoleucine (I). In contrast, one had histidine (H) at residue 10 even though this residue spot is occupied by leucine (L) on the 3D7 reference strain. All but one of the present isolates translated phenylalanine (F) at position 19 as opposed to leucine on the 3D7 reference strain. Isolate DM001 had near absolute amino acid array similarity to 3D7 reference. The only variance that occurred at position 11

and 22 were these isolate registered leucine and aspartic acid instead of serine and asparagine, as seen on the reference strain.

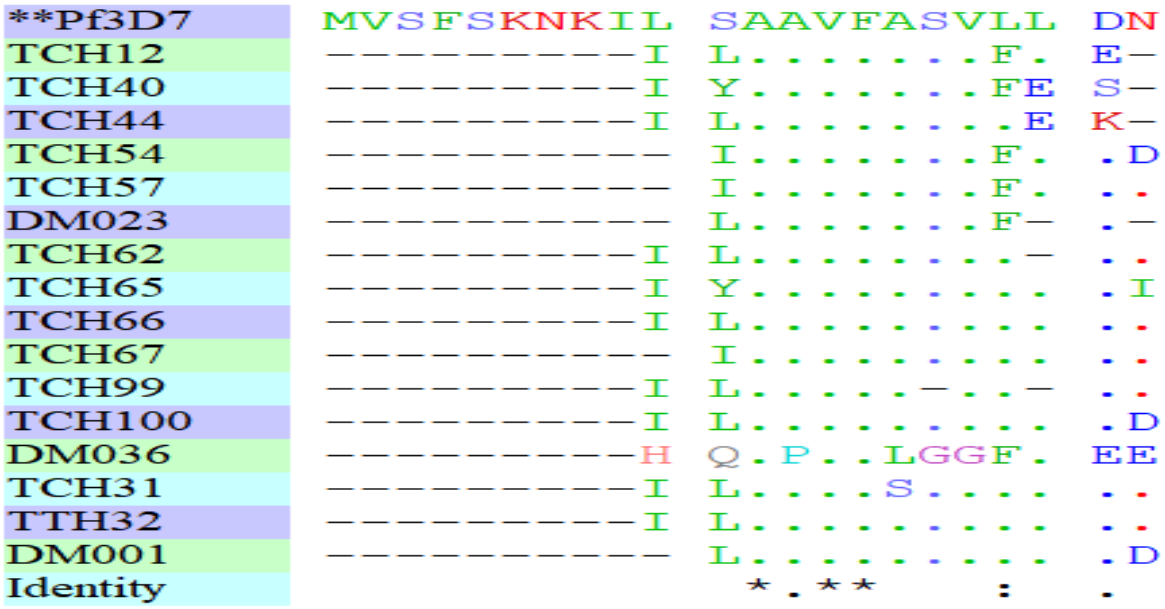


Figure 4.10: Multiple sequence alignment of translated *Pfhrp2*–exon1 residues of present study to *P. falciparum* 3D7 reference strain.

* means 100 % identity at the particular residue site.

4.6.2 Haplotype Analysis of *Plasmodium falciparum* histidine-rich protein *3*–Intron

All sixteen isolates were distinct haplotypes (Figure 4.11), indicating that there were no two identical intron- sequences among the present study isolates, just as the exon sequences depicted. Haplotype analysis showed gene diversity occurrence of 100 % across all sites, 38 variable sites (S), 42 total mutations (Eta) across variable sites and nucleotide diversity (π) of 0.13517.

The 16 haplotypes had a dispersive network with 2 – 11 mutational steps between neighbouring haplotypes. The network also revealed six hypothetical haplotypes that were not found among isolates used in the present study. However, nine peripheral haplotypes DM023, TCH99, TCH40, TCH57, DM001, TCH31, TCH100, TCH54 and TCH12 (Figure 4.11) are likely to be newer of the 16 isolates indicating a recent evolutionary pattern. Carriers have not had evolutionary time to disperse and breed across the *P. falciparum* population so far as the histidine-rich protein 3 gene is concerned.

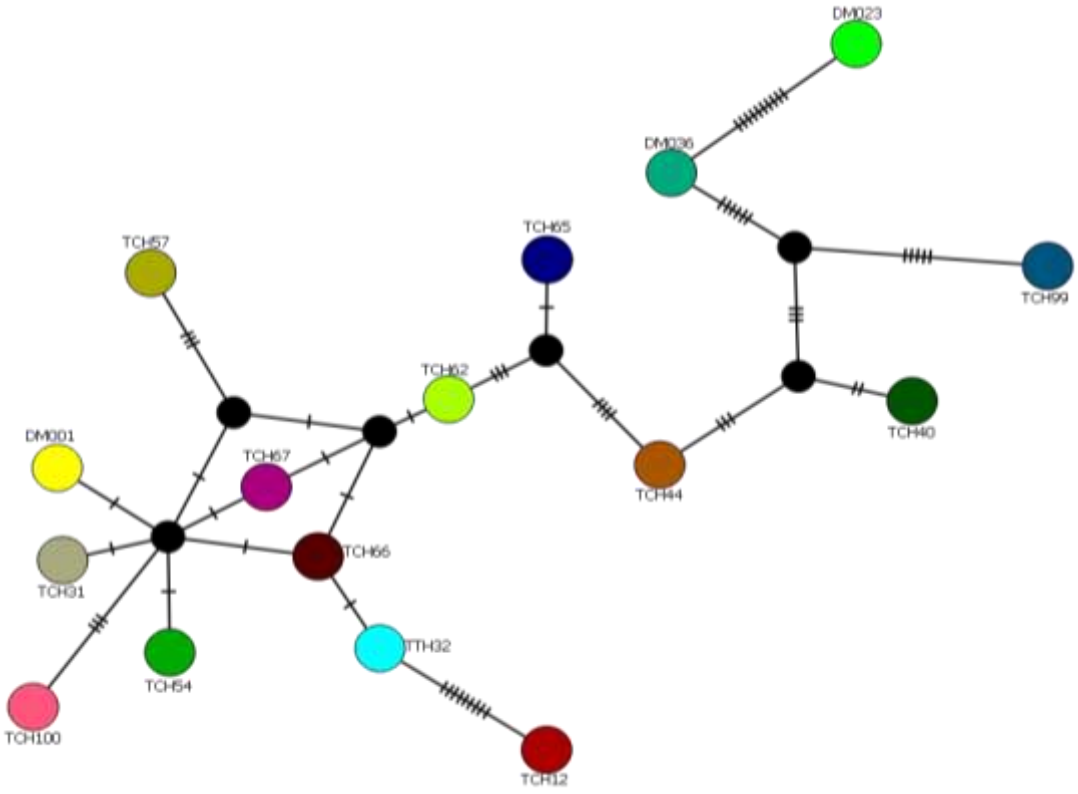


Figure 4.11: Haplotype network of *Pfhrp*3 (Introns) sequences indicating variabilities between isolates of the present study.

Mutational steps are shown by number of hatch marks between vertices. The black dots/circles are hypothetical haplotypes not seen among isolates of the present study.

4.6.3 Phylogenetic Analysis of *Plasmodium falciparum* histidine-rich protein 3 (*Pfhrp*3) Gene Sequences

The drawn Maximum Likelihood (ML) Phylogenetic tree (Figure 4.12) indicated 20 nucleotide substitutions per 100 bp of isolates involved in the phylogeny analysis. Based on the *Pfhrp3* sequenced data, 10 out of the 16 isolates formed a monophyletic clade at the base of the tree, indicating that they are earlier ancestors to all other sequences employed in the tree analysis except for PfNF54 West Africa strain. Although TCH54, TCH57, TCH100, TCH31 and DM001 of the present study clade monophyletically, all 16 isolates of Ghanaian descent are only paraphyletically related to the WHO's *P. falciparum* 3D7 reference strain. Divergence of the TCH66 isolate is the midpoint of the two cluster groups of the isolates.

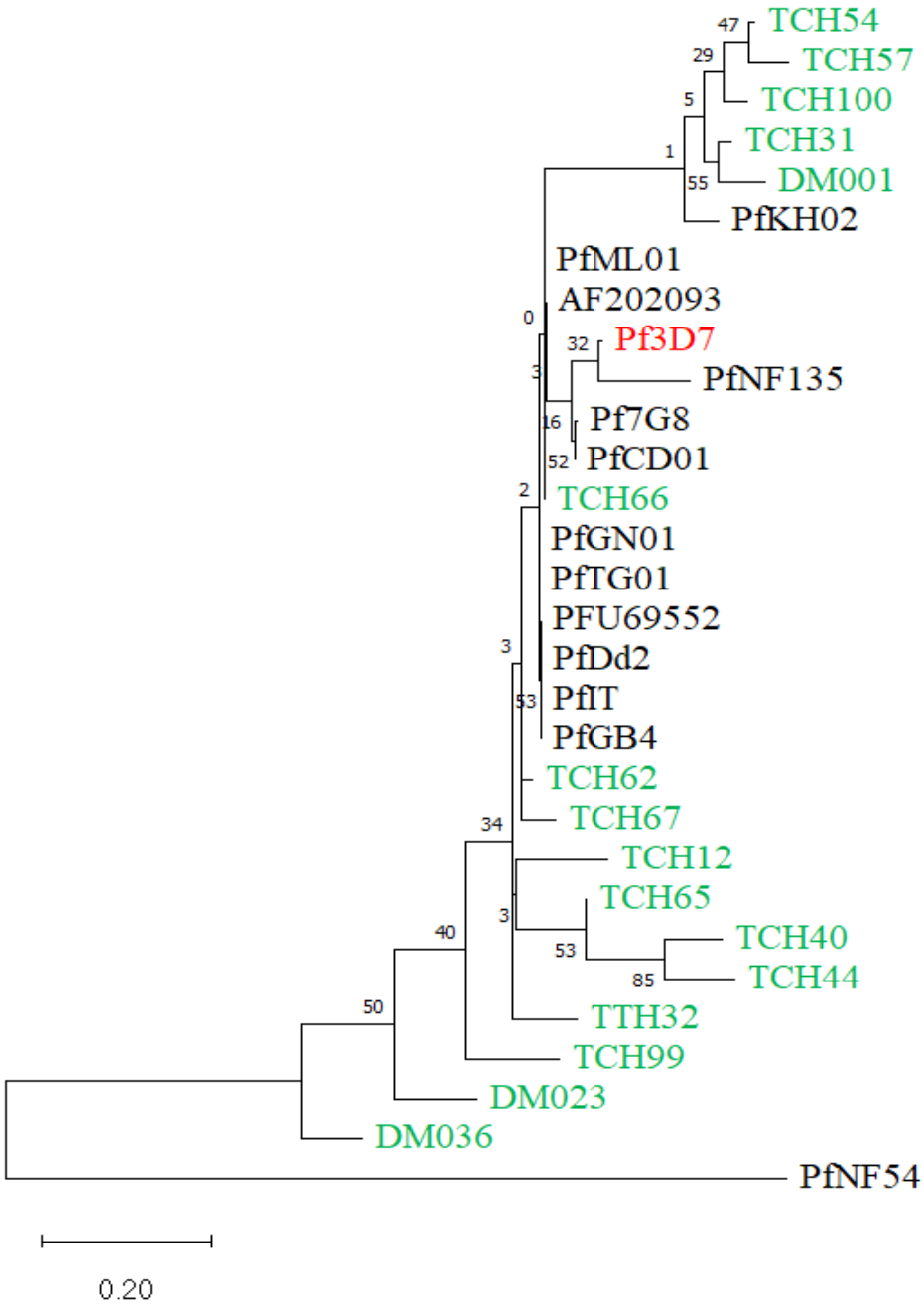


Figure 4.12: Phylogenetic tree of *P. falciparum* isolates based on the *Pfhrp*3 gene sequences of the present study analyzed with referenced **deposits.**

Sequences of the present study are shown in green font while the 3D7 reference strain is in red font.

CHAPTER FIVE

5.0 DISCUSSION

Currently, the use of malaria rapid diagnostic tests (RDTs) is widely accepted globally because of its suitability and convenience. In sub-Saharan Africa, RDTs, especially those based on *Pf*HRP2 antigen detection, have and continue to be pivotal in malaria control programmes (Berzosa et al., 2018; Funwei et al., 2019; Grignard et al., 2020; Kojom and Singh, 2020; Shehu et al., 2021). Nevertheless, they are not error-proof and have issues that need to be addressed to improve healthcare in the region.

In the present study, more than 40 % of microscopy-confirmed malaria blood samples taken from patients from the northern part of Ghana yielded no results for either the *P. falciparum*-specific PCR or the *Pf*HRP2-RDT. This may be the case that other *Plasmodium* species are responsible for the malaria diagnosis by microscopy as four other *Plasmodium* species are aetiological agents of human malaria (Gerstl et al., 2010; WHO, 2019b). To elucidate the possible role of *Plasmodium* species other than *P. falciparum* in malaria in the study area, microscopic screening showed that two (2) samples were infected with *P. malariae* of which one showed no amplification for the *P. falciparum*-specific PCR. Although *P. vivax* and *P. knowlesi* are reportedly uncommon in sub-Saharan Africa, up to 15 namely Angola, Benin, Cameroon, Congo Comoros, Eritrea, Equatorial Guinea, Kenya, Mauritania, Madagascar, Mali, Senegal, Uganda, Zambia and Zimbabwe have reported the detection of *P. vivax* in clinical samples (Rubio et al., 1999; Guerra-neira et al., 2006; Robert et al., 2006; Ryan et al., 2006; Ménard et al., 2010; Mendes et al., 2011; Wurtz et al., 2011; Fru-Cho et al., 2014; Mbenda and Das, 2014; Salem et al., 2015; Ba et al., 2016; Moukah et al., 2016; Mze

et al., 2016; Poirier et al., 2016; Berhane et al., 2017; Russo et al., 2017; Berzosa et al., 2018; Brazeau et al., 2018; Kavunga-Membo et al., 2018)

Besides *P. falciparum* which is the dominant malaria parasite, there have been reports of the occurrences of non-falciparum malaria infections in Ghana as well. Bredu and colleagues reported a combined prevalence of *P. malariae* and *P. ovale* malaria to be 29.8 % in asymptomatic children from the Central Region of Ghana based on PCR detection (Bredu et al., 2021). Another study in Kwahu-South District of Ghana reported a 12.7 % prevalence of *P. malariae* (Owusu et al., 2017). It is important to understand the distribution of *Plasmodium* species other than *P. falciparum* in Ghana to be able to assess their impact on malaria diagnosis and control programmes. The present data concerning the prevalence of possible non-falciparum malaria parasites further points to the need for a broad appraisal of the distribution of these parasites in Ghana and the use of *Pf*HRP2-RDT in malaria diagnosis in the country.

While the performances of *Pf*HRP2-RDT and *Pf* specific-PCR in detecting *P. falciparum* infection appear to be similar (59.2 % and 59.9 %, respectively), the results revealed rather high RDT false positive (30.8 %) and false negative (21.9 %) rates. These figures are however consistent with results from a recent study in Ghana by Amoah et al. (2019) who reported *Pf*HRP2-RDT false positive figures to be 10 % in adults aged above 20 years and 64.6 % in children between 5 – 9 years old (Amoah et al., 2019). Earlier reports have recorded *Pf*HRP2-RDT positives about 5 – 32 days (Dalrymple et al., 2018) and 35 – 42 days (Grandesso et al., 2016) post malaria treatment. The false positive results in the present study may be attributed to existing antigenic protein in the blood of the patient from a recent past malaria infection even

though this possibility was not investigated in the present study. Again, non-Plasmodium infectious agents and immunological factors can trigger the occurrence of false RDT positives (Lee et al., 2014). Immunologic cross-reaction from heterophile antigens produced by hepatitis C virus, dengue virus and *Toxoplasma gondii* are reported to be contributing factors to false positivity of *Pf*HRP2-RDT (Maltha et al., 2013; Lee et al., 2014; Haberichter et al., 2017; Gatton et al., 2018). There is also the possibility of faulty RDT kits in this context which may arise as a result of suboptimal storage conditions (Orish et al., 2018; World Health Organization (WHO), 2019b).

The occurrence of false RDT negative results in malaria diagnosis, especially in resource-deprived settings, has dire consequence in the disease management and control if other diagnostic tools such as microscopy are not available. A reduced sensitivity of RDT, as high as 70 % compared with PCR, and an overall 62 % RDT false negative rate have been reported in Eretria (Berhane et al., 2018). Several factors can account for the reduced performance of RDT, such as low parasite density, low HRP antigen concentration, very high parasite load which can cause prozone effect, and the deletion and/or microvariations of the *Pfhrp2* and/or *3* gene(s) in the *P. falciparum* parasite (Luchavez, et al., 2011; Maltha et al., 2013; Orish et al., 2018; WHO, 2019b; Amoah et al., 2020). The clinical risk associated with false-negative RDT results due to prozone effect or hyper-parasitaemia may be mitigated by repeated testing after an unexpected negative RDT result (Gillet et al., 2011). The HRP2 antigen concentration was not determined in the present study but the false RDT negatives were recorded in blood samples with high and low parasitic load at par. Also, *Pf*HRP2-RDT false negative results could be caused by poor technical quality of the test kit. Exposure of RDT kits

to high humidity and temperature during transportation and in storage can lead to degradation of the antibody and therefore affect its reliability and reduce diagnostic performance (Bakari et al., 2020; King et al., 2021).

The molecular assessment of *Pfhrp*2 (exon 1–2) gene revealed a deletion of the gene in 11.9 % of PCR-confirmed *P. falciparum* isolates. This is beyond the 5 % threshold set by the WHO pertaining to RDT target marker deletion causing false RDT negative results implying that malaria programmes will have to switch to RDTs that do not rely exclusively on HRP2 of *P. falciparum.* The target gene deletion was first recorded in the Amazon Basin in Peru in 2010 where about 40 % of *P. falciparum* isolates lacked the *Pfhrp2* gene (Gamboa et al., 2010). Later, several reports of the deletion of either exon1–2 or Exon 2 or both in sampled isolates were made from Southern America and Asia (Murillo et al., 2015; Bharti et al., 2016; Fontecha et al., 2018) . Africa is no exception as 6.4 %, 62.0 %, 9.0 % and 23.0 % of *P. falciparum* isolates recorded *Pfhrp2* gene deletions in Democratic Republic of Congo, Eritrea, Kenya and Rwanda, respectively (Beshir et al., 2017; Kozycki et al., 2017; Parr et al., 2017 Berhane et al., 2018;). In 2020, Amoah et al. reported deletion of *Pfhrp2* exon1–2 in 12.9 % of isolates and deletions in exon2 only in 39.5 % of isolates in southern Ghana (Amoah et al., 2020). An unpublished report from the Kassena-Nankana Districts of Upper East Region, (northern Ghana), indicated 11.1 % of isolates to have deletion of *Pfhrp2* gene (Ayelazuno, 2017). The target marker for CareStart™ Malaria *Pf*HRP2-RDT is the *P. falciparum* histidine-rich protein 2 (*Pfhrp2*). However, reports that the test strips of RDT kits which contains MAbs are able to cross-react with antigenic proteins expressed by another homologue HRP gene family, *Pfhrp3*. And this is possible because of the strong

amino acid sequence similarity (Wellems and Howard, 1986; Gatton et al., 2018; Nyataya et al., 2020). For this reason, it is advised that both genes be detected in such assays. Owing to the homology of *Pfhrp2* gene to *Pfhrp3* gene, *the* WHO recommends that confirmatory evidence of *Pfhrp* gene deletions must include both genes. In the present study, 13.8 % of PCR-confirmed *P. falciparum* samples lacked exon1–2 of *Pfhrp3*. A similar rate was recorded in a cross-sectional study in all regions of Ghana where 15.2 % of isolates had deletion of the *Pfhrp3* (exon1–2) as well as 40.5 % of isolates with deletion of the exon2 in same study (Amoah et al., 2020). In the Upper East Region, 1 % of isolates were reported with deletion of the exon2 section of *Pfhrp3* gene (Ayelazuno, 2017). Even though the analysis of the present study targeted exon1–2 of the genes, prevalence analysis of deletion patterns of exon1–2 and exon2 generally shows an increased rate for exon2 than exon1−2 (Parr et al., 2017; Berhane et al., 2018; Amoah et al., 2020). *Plasmodium falciparum*, isolates that lacked both *Pfhrp*2 and 3 genes were 10.6 % in present study compared to 27.5 % reported earlier in southern Ghana (Amoah et al., 2016). Around 18.0 % of *P. falciparum* isolates were recorded to have both genes deleted in Eritrea (Berhane et al., 2018). Out of the 35 samples that were false negative by *Pf*HRP2-RDT, 16 (45.7 %) lacked the exon1–2 in both *Pfhrp*2 and 3 while 1 (2.9 %) and 3 (8.6 %) lacked the sequence in only *Pfhrp*2 and only *Pfhrp*3, respectively. The 20 false RDT negative results are most likely attributed to the lack of the target a HRP2 protein and its HRP3 homologue. In the present study, half of the isolates that were *Pfhrp*2*-/Pfhrp*3+ tested positive for *Pf*HRP2-RDT confirming that there is cross reactivity between HRP2 monoclonal antibodies and HRP3 antigen. This is an important attribute that helps mitigate the misdiagnosis associated with *Pfhrp2*

gene deletion by playing a compensatory role in the absence of the functional *Pfhrp2* (Lee et al., 2006; Baker et al., 2010; Lee et al., 2012; WHO, 2019b). The other half that showed no cross reaction could be due to mutation of the *Pfhrp*3 gene that produces a faulty protein or due to its lower expression (Poti et al., 2020).

Genetic microvariation of the *Pf*HRP2-RDT target antigen for malaria diagnosis and *P. falciparum* in general, has consequences on malaria control and management studies. Although specificity of the RDT monoclonal antibodies largely depend on exon2-based epitope of the *hrp*2 antigen which the present study did not amplify, a plausible inference can be made from the observed microvariation of exon1–2 of the *Pfhrp*2 and 3 genes that this exon is as well mutated in the isolates. Impact analysis of the microvariation of exon1–2 of *Pfhrp*2 and 3 genes has not been appraised in this study, but the genetics, haplotype and phylogenetic indices of sequenced fragment of target gene in the present study showed higher variation especially when analyses are compared to reference strains. A closer look at the genetic analyses suggests that the parasites of the current study diverged on the premise of local selective pressures. This is because isolates mostly cluster among themselves and away from other isolates including the reference *P. falciparum* 3D7. This observation is supported by a similar report by (Berhane et al., 2017; Nyataya et al., 2020) in studies conducted in Eritrea (Berhane et al., 2017).

Study that explores the variation within the exon2 of the antigenic marker on a larger population scale is necessary to understand the extent of gene deletion or mutation in *P. falciparum* isolates in the northern regions of Ghana. This is important as malaria

control programmes and policies in the country should be informed by comprehensive local epidemiological data on *Plasmodium* species diversity and evolutionary biology.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Microscopy remains the gold standard for malaria diagnosis in Ghana as the widely used *Pf*HRP2-RDT has been shown in this study to have reduced reliability in the diagnosis of the disease. While the performance of *Pf*HRP2-RDT was comparable to *P. falciparum* 18S rRNA PCR, the high rate of false RDT negative and false RDT positive results pose a serious challenge to malaria diagnosis in the study area. Even though the cause of the false positive *Pf*HRP2-RDT results was not investigated in this study, our findings show that the poor performance *Pf*HRP2-RDT results may be due to the increasing prevalence of non-falciparum malaria species or by mutations in the Pfhrp2 and/ 3 gene leading to the production of an undetectable antigen. The former is particularly plausible as more than 40 % of microscopy-confirmed malaria blood samples taken from patients from the northern part of Ghana yielded no results for the *P. falciparum*-specific PCR or the *Pf*HRP2-RDT. Concerning possible mutation of Pfhrp2 and/ 3, our results revealed significant mutations to either or both of the *Pfhrp*2, and *Pfhrp*3 genes. Though preliminary, the trends revealed in our study put together raise serious questions about the reliability of the use of *Pf*HRP2/3-based RDTs for the diagnosis of malaria in northern Ghana.

Genetic and evolutionary analysis of both the *Pfhrp*2 and *Pfhrp*3 genes showed a higher and earlier divergence of present study isolates in comparison with other reference and non-reference strains which is most likely due to a local selective pressure in the study areas and may impact on the overall stability of target genes for malaria RDTs.

6.2 Recommendation

i. These preliminary results recommend the use of malaria RDTs that detect all *Plasmodium* species as *Pf*HRP2-RDT has been shown in this study to produce high false negative and false positive RDT results in the study area. This will reduce the chances of misdiagnoses and its impact on malaria management in Ghana especially in resource-deprived settings.

ii. Exon2 of *Pfhrp*2 and 3 genes of the study isolates should be amplified, sequenced and analyzed to determine the extent of mutation of the target of *Pf*HRP2-RDT.

iii. Further molecular studies should be conducted to investigate the prevalence of non-falciparum malaria species in the study area.

REFERENCES

Abdallah, J. F., Okoth, S. A., Fontecha, G. A., Mejia Torres, R. E., Banegas, E. I., Matute, M. L., Bucheli, S. T. M., Goldman, I. F., de Oliveira, A. M., Barnwell, J. W., & Udhayakumar, V. (2015). Prevalence of *Pfhrp2* and *Pfhrp3* gene deletions in Puerto Lempira, Honduras. *Malaria Journal*, *14*(1). https://doi.org/10.1186/s12936-014-0537-7

Abdelwhab, O. F., Elaagip, A., Albsheer, M. M., Ahmed, A., Paganotti, G. M., & Abdel Hamid, M. M. (2021). Molecular and morphological identification of suspected *Plasmodium vivax* vectors in Central and Eastern Sudan. *Malaria Journal*, *20*(1), 1–7. https://doi.org/10.1186/s12936-021-03671-9

Abukari, Z., Okonu, R., Nyarko, S. B., Lo, A. C., Dieng, C. C., Salifu, S. P., Gyan, B. A., Lo, E., & Amoah, L. E. (2019). The diversity, multiplicity of infection and population structure of P. falciparum parasites circulating in asymptomatic carriers living in high and low malaria transmission settings of Ghana. *Genes*, *10*(6). https://doi.org/10.3390/genes10060434

Addai-Mensah, O., Dinko, B., Noagbe, M., Ameke, S. L., Annani-Akollor, M. E., Owiredu, E. W., Mensah, K., Tackie, R., Togbe, E., Agyare-Kwabi, C., Gyasi, C., Adu-Gyamfi, C., & Debrah, A. Y. (2020). *Plasmodium falciparum* histidine-rich protein 2 diversity in Ghana. *Malaria Journal*, *19*(1). https://doi.org/10.1186/s12936-020-03328-z

Adu-Gyasi, D., Asante, K. P., Amoako, S., Amoako, N., Ankrah, L., Dosoo, D., Tchum, S. K., Adjei, G., Agyei, O., Amenga-Etego, S., & Owusu-Agyei, S. (2018). Assessing the performance of only HRP2 and HRP2 with pLDH based rapid diagnostic tests for the diagnosis of malaria in middle Ghana, Africa. *PLoS ONE*, *13*(9), 1–13. https://doi.org/10.1371/journal.pone.0203524

Agaba, B. B., Yeka, A., Nsobya, S., Arinaitwe, E., Nankabirwa, J., Opigo, J., Mbaka, P., Lim, C. S., Kalyango, J. N., Karamagi, C., & Kamya, M. R. (2019). Systematic review of the status of pfhrp2 and pfhrp3 gene deletion, approaches and methods used for its estimation and reporting in Plasmodium falciparum populations in Africa: Review of published studies 2010-2019. In *Malaria Journal* (Vol. 18, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s12936-019-2987-4

Al-Awadhi, M., Ahmad, S., & Iqbal, J. (2021). Current status and the epidemiology of malaria in the Middle east region and beyond. *Microorganisms*, *9*(338), 1–20. https://doi.org/10.3390/microorganisms9020338

Alemayehu, G. S., Lopez, K., Dieng, C. C., Lo, E., Janies, D., Golassa, L., & Carolina, N. (2020). Evaluation of *Pf*HRP2 and *Pf*LDH Malaria Rapid Diagnostic Test Performance in Assosa Zone, Ethiopia. *The American Society of Tropical Medicine and Hygiene*, *103*(5), 1902–1909. https://doi.org/10.4269/ajtmh.20-0485

Amir, A., Cheong, F. W., Ryan De Silva, J., Wee, J. K., & Lau, Y. L. (2018). *Plasmodium knowlesi* malaria: current research perspectives. *Infection Drug Resistance*, 1145–1155.

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. *Malaria Journal*, *15*(1). https://doi.org/10.1186/s12936-016-1159-z

Amoah, L. E., Abuaku, B., Bukari, A. H., Dickson, D., Amoako, E. O., Asumah, G., Asamoah, A., Preprah, N. Y., & Malm, K. L. (2020). Contribution of *P. falciparum* parasites with *Pfhrp2* gene deletions to false negative *Pf*HRP2-based malaria RDT results in Ghana: A nationwide study of symptomatic malaria patients. *PLoS ONE*, *15*(9 September). https://doi.org/10.1371/journal.pone.0238749

Amoah, L. E., Donu, D., Abuaku, B., Ahorlu, C., Arhinful, D., Afari, E., Malm, K., & Koram, K. A. (2019). Probing the composition of *Plasmodium* species contained in malaria infections in the Eastern region of Ghana. *BMC Public Health*, *19*(1), 1–11. https://doi.org/10.1186/s12889-019-7989-1

AngloGold Ashanti. (2018). *AGA Malaria and Public-Private Partnerships in Ghana's Health Sector: a case study*.

Antinori, S., Galimberti, L., Milazzo, L., & Corbellino, M. (2013). *Plasmodium knowlesi*: The emerging zoonotic malaria parasite. *Acta Tropica*, *125*(2), 191–201. https://doi.org/10.1016/j.actatropica.2012.10.008

Awuah, R. B., Asante, P. Y., Sakyi, L., Biney, A. A. E., Kushitor, M. K., Agyei, F., & De-Graft Aikins, A. (2018). Factors associated with treatment-seeking for malaria

in urban poor communities in Accra, Ghana. *Malaria Journal*, *17*(1), 1–8. https://doi.org/10.1186/s12936-018-2311-8

Ayelazuno, F. A. (2017). *Analysis of histidine-rich protein 2 and 3 gene deletion polymorphisms In Northern Ghana*. University of Ghana.

Ba, H., Duffy, C. W., Ahouidi, A. D., Deh, Y. B., Diallo, M. Y., Tandia, A., & Conway, D. J. (2016). Widespread distribution of *Plasmodium vivax* malaria in Mauritania on the interface of the Maghreb and West Africa. *Malaria Journal*, *15*(80), 1–6. https://doi.org/10.1186/s12936-016-1118-8

Bailey, J. W., Williams, J., Bain, B. J., Parker-Williams, J., & Chiodini, P. L. (2013). Guideline: The laboratory diagnosis of malaria. *British Journal of Haematology*, *163*(5), 573–580. https://doi.org/10.1111/bjh.12572

Baird, J. K., & Hoffman, S. L. (2004). Primaquine therapy for malaria. *Clinical Infectious Diseases*, *39*(9), 1336–1345. https://doi.org/10.1086/424663

Bakari, C., Jones, S., Subramaniam, G., Mandara, C. I., Chiduo, M. G., Rumisha, S., Chacky, F., Molteni, F., Mandike, R., Mkude, S., Njau, R., Herman, C., Nace, D. P., Mohamed, A., Udhayakumar, V., Kibet, C. K., Nyanjom, S. G., Rogier, E., & Ishengoma, D. S. (2020). Community-based surveys for *Pfhrp2* and *Pfhrp3* gene deletions in selected regions of mainland Tanzania. In *medRxiv*. https://doi.org/10.1101/2020.05.12.20097766

Baker, J., Ho, M., Pelecanos, A., Gatton, M., Chen, N., Abdullah, S., Albertini, A., Ariey, F., Barnwell, J., Bell, D., Cunningham, J., & Djalle, D. (2010). Global sequence variation in the histidine-rich proteins 2 and 3 of *Plasmodium falciparum*: implications for the performance of malaria rapid diagnostic tests. *Malaria Journal*, *9*(129), 1–12.

Baker, J., Mccarthy, J., Gatton, M., Kyle, D. E., Belizario, V., Luchavez, J., Bell, D., & 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. *The Journal of Infectious Diseases*, *2*, 1–8.

Barredo, E., & DeGennaro, M. (2020). Not Just from Blood: Mosquito Nutrient Acquisition from Nectar Sources. *Trends in Parasitology*, *36*(5), 473–484. https://doi.org/10.1016/j.pt.2020.02.003

Bereczky, S., Mårtensson, A., Gil, J. P., & Farnert, A. (2005). Short Report: Rapid DNA Extraction from Archive Blood Spots on Filter Paper for Genotyping of *Plasmodium falciparum*. In *J. Trop. Med. Hyg* (Vol. 72, Issue 3).

Berhane, A., Anderson, K., Mihreteab, S., Gresty, K., Rogier, E., Mohamed, S., Hagos, F., Embaye, G., Chinorumba, A., Zehaie, A., Dowd, S., Waters, N. C., Gatton, M. L., Udhayakumar, V., Cheng, Q., & Cunningham, J. (2018). Major threat to malaria control programs by Plasmodium falciparum lacking histidine-rich protein 2, Eritrea. *Emerging Infectious Diseases*, *24*(3), 462–470. https://doi.org/10.3201/eid2403.171723

Berhane, A., Russom, M., Bahta, I., Hagos, F., Ghirmai, M., & Uqubay, S. (2017). Rapid diagnostic tests failing to detect *Plasmodium falciparum* infections in Eritrea: an investigation of reported false negative RDT results. *Malaria Journal*, *16*(105), 1–6. https://doi.org/10.1186/s12936-017-1752-9

Berzosa, P., de Lucio, A., Romay-Barja, M., Herrador, Z., González, V., García, L., Fernández-Martínez, A., Santana-Morales, M., Ncogo, P., Valladares, B., Riloha, M., & Benito, A. (2018). Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea 11 Medical and Health Sciences 1108 Medical Microbiology. *Malaria Journal*, *17*(1), 1–12. https://doi.org/10.1186/s12936-018-2481-4

Berzosa, P., González, V., Taravillo, L., Mayor, A., Romay-Barja, M., Garciá, L., Ncogo, P., Riloha, M., & Benito, A. (2020). First evidence of the deletion in the *Pfhrp2* and *Pfhrp3* genes in *Plasmodium falciparum* from Equatorial Guinea. *Malaria Journal*, *19*(1). https://doi.org/10.1186/s12936-020-03178-9

Beshir, K. B., Sepúlveda, N., Bharmal, J., Robinson, A., Mwanguzi, J., Busula, A. O., de Boer, J. G., Sutherland, C., Cunningham, J., & Hopkins, H. (2017). *Plasmodium falciparum* parasites with histidine-rich protein 2 (*Pfhrp2*) and *Pfhrp3* gene deletions in two endemic regions of Kenya. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-15031-2

Bharti, P. K., Chandel, H. S., Ahmad, A., Krishna, S., Udhayakumar, V., & Singh, N. (2016). Prevalence of *Pfhrp2* and/or *Pfhrp3* gene deletion in *P. falciparum* population in eight highly endemic states in India. *PLoS ONE*, *11*(8). https://doi.org/10.1371/journal.pone.0157949

Bharti, P. K., Chandel, H. S., Krishna, S., Nema, S., Ahmad, A., Udhayakumar, V., & Singh, N. (2017). Sequence variation in *Plasmodium falciparum* histidine rich proteins 2 and 3 in Indian isolates: implications for malaria rapid diagnostic test performance. *Scientific Reports*, *7*(10). https://doi.org/10.1038/s41598-017-01506-9

Biggs, B. A., Gooze, L., Wycherley, K., Southwell, B., Leech, J. H., & Brown, G. V. (1991). Antigenic variation in *Plasmodium falciparum*. *Proc. Natl. Acad. Science*, *88* (October), 9171–9174.

Bogitsh, B. J., Carter, C. E., & Oeltmann, T. N. (2019). Blood and Tissue Protistans III. In *Human Parasitology* (pp. 111–133). https://doi.org/10.1016/b978-0-12-813712-3.00007-2

Bortier, E. (2019). *Prevalence of Pfhrp2 and/or Pfhrp3 gene deletions in Plasmodium falciparum isolates and the performance of hrp2 based malaria rapid diagnostic tests*. University of Ghana, Legon.

Boyce, M. R., & Meara, W. P. O. (2017). Use of malaria RDTs in various health contexts across sub-Saharan Africa: a systematic review. *BMC Public Health*, *17*(470), 1–15. https://doi.org/10.1186/s12889-017-4398-1

Brasil, P., De Pina Costa, A., Pedro, R. S., Da Silveira Bressan, C., Da Silva, S., Tauil, P. L., & Daniel-Ribeiro, C. T. (2011). Unexpectedly long incubation period of *Plasmodium vivax* malaria, in the absence of chemoprophylaxis, in patients diagnosed outside the transmission area in Brazil. *Malaria Journal*, *10*, 1–5. https://doi.org/10.1186/1475-2875-10-122

Brazeau, N. F., Whitesell, A. N., Doctor, S. M., Keeler, C., Mwandagalirwa, M. K., Tshefu, A. K., Likwela, J. L., Juliano, J. J., & Meshnick, S. R. (2018). *Plasmodium vivax* Infections in Duffy-Negative Individuals in the Democratic Republic of the Congo. *Am. J. Trop. Med. Hyg*, *99*(5), 1128–1133. https://doi.org/10.4269/ajtmh.18-0277

Bredu, D., Donu, D., & Amoah, L. E. (2021). Dynamics of the Composition of *Plasmodium* Species Contained within Asymptomatic Malaria Infections in the Central Region of Ghana. *Journal of Tropical Medicine*, *2021*. https://doi.org/10.1155/2021/7419548

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. *Malaria Journal*, *15*(1), 1–8. https://doi.org/10.1186/s12936-016-1158-0

Bruce-Chwatt, L. J., Wernsdorfer, W., & McGregor, I. (1988). History of Malaria from Prehistory to Eradication. *Malaria: Principles and Practice of Malariology*, 1–59.

Chima, R. I., Goodman, C. A., & Mills, A. (2003). The economic impact of malaria in Africa: a critical review of the evidence. *Health Policy*, *63*(1), 17–36. https://doi.org/10.1016/S0168-8510(02)00036-2

Cibulskis, R. E., Alonso, P., Aponte, J., Aregawi, M., Barrette, A., Bergeron, L., Fergus, C. A., Knox, T., Lynch, M., Patouillard, E., Schwarte, S., Stewart, S., & Williams, R. (2016). Malaria: Global progress 2000 – 2015 and future challenges. *Infectious Diseases of Poverty*, *5*(61), 1–8. https://doi.org/10.1186/s40249-016-0151-8

Clement, M., Snell, Q., Walker, P., Posada, D., & Crandall, K. (2002). TCS: Estimating gene genealogies. *Parallel and Distributed Processing Symposium, International Proceedings*, *2*, 184. https://doi.org/10.1109/IPDPS.2002.1016585

Cnops, L., Boderie, M., Gillet, P., Van Esbroeck, M., & Jacobs, J. (2011). Rapid diagnostic tests as a source of DNA for *Plasmodium* species-specific real-time PCR. *Malaria Journal*, *10*. https://doi.org/10.1186/1475-2875-10-67

Coetzee, M., Hunt, R. H., Wilkerson, R., Della, A., Coulibaly, M. B., & Besansky, N. J. (2013). *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. *Zootaxa*, *3*(February), 246–274. https://doi.org/10.11646/zootaxa.3619.3.2

Collins, W. E., & Jeffery, G. M. (2007). *Plasmodium malariae*: Parasite and disease. *Clinical Microbiology Reviews*, *20*(4), 579–592. https://doi.org/10.1128/CMR.00027-07

Cox, F. E. G. (2010). History of the discovery of the malaria parasites and their vectors. *Parasites and Vectors*, *9*(1), 1–8. https://doi.org/10.4018/jec.2013010101

Cunningham, J., Jones, S., Gatton, M. L., Barnwell, J. W., Cheng, Q., Chiodini, P. L., Glenn, J., Incardona, S., Kosack, C., Luchavez, J., Menard, D., Nhem, S., Oyibo, W., Rees-Channer, R. R., Gonzalez, I., & Bell, D. (2019). A review of the WHO malaria rapid diagnostic test product testing programme (2008-2018): Performance, procurement and policy. *Malaria Journal*, *18*(387). https://doi.org/10.1186/s12936-019-3028-z

Da Silva, G. B. J., Pinto, J. R., Barros, E. J. G., Farias, G. M. N., & Daher, E. D. F. (2017). Kidney involvement in malaria: An update. *Journal of the Sao Paulo Institute of Tropical Medicine*, *59*(December 2016), 1–6. https://doi.org/10.1590/S1678-9946201759053

Dalrymple, U., Arambepola, R., Gething, P. W., & Cameron, E. (2018). How long do rapid diagnostic tests remain positive after anti-malarial treatment? *Malaria Journal*, *17*(1), 1–13. https://doi.org/10.1186/s12936-018-2371-9

Dinko, B., Oguike, M. C., Larbi, J. A., Bousema, T., & Sutherland, C. J. (2013). Persistent detection of *Plasmodium falciparum, P. malariae, P. ovale curtisi* and *P. ovale wallikeri* after ACT treatment of asymptomatic Ghanaian school-children. *International Journal for Parasitology: Drugs and Drug Resistance*, *3*, 45–50. https://doi.org/10.1016/j.ijpddr.2013.01.001

Ebrahimi, B., Jackson, B. T., Guseman, J. L., Przybylowicz, C. M., Stone, C. M., & Foster, W. A. (2018). Alteration of plant species assemblages can decrease the transmission potential of malaria mosquitoes. *Journal of Applied Ecology*, *55*(2), 841–851. https://doi.org/10.1111/1365-2664.13001

Feachem, R., & Sabot, O. (2008). Viewpoint A new global malaria eradication strategy. *Lancet Infectious Disease*, *371*, 1633–1635. https://doi.org/10.1016/S0140-6736(08)60424-9

Fidock, D. A., & Rosenthal, P. J. (2021). Artemisinin resistance in Africa: How urgent is the threat? *Med*, *2*(12), 1287–1288. https://doi.org/10.1016/j.medj.2021.11.005

Fontecha, G., Mejía, R. E., Banegas, E., Ade, M. P., Mendoza, L., Ortiz, B., Sabillón, I., Alvarado, G., Matamoros, G., & Pinto, A. (2018). Deletions of *Pfhrp2* and *Pfhrp3* genes of *Plasmodium falciparum* from Honduras, Guatemala and Nicaragua. *Malaria Journal*, *17*(1). https://doi.org/10.1186/s12936-018-2470-7

Fru-cho, J., Bumah, V. V, Safeukui, I., Nkuo-akenji, T., Titanji, V. P. K., & Haldar, K. (2014). Molecular typing reveals substantial *Plasmodium vivax* infection in asymptomatic adults in a rural area of Cameroon. *Malaria Journal*, *13*(170), 1–11.

Fuehrer, H. P., & Noedl, H. (2014). Recent advances in detection of *Plasmodium ovale*: Implications of separation into the two species *P. ovale wallikeri* and *P. ovale curtisi*. *Journal of Clinical Microbiology*, *52*(2), 387–391. https://doi.org/10.1128/JCM.02760-13

Fuehrer, H. P., Starzengruber, P., Swoboda, P., Ali Khan, W., Matt, J., Ley, B., Thriemer, K., Haque, R., Yunus, E. Bin, Hossain, S. M., Walochnik, J., & Noedl, H. (2010). Indigenous *Plasmodium ovale* malaria in Bangladesh. *American Journal of Tropical Medicine and Hygiene*, *83*(1), 75–78. https://doi.org/10.4269/ajtmh.2010.09-0796

Funwei, R., Nderu, D., Nguetse, C. N., Thomas, B. N., Falade, C. O., Velavan, T. P., & Ojurongbe, O. (2019). Molecular surveillance of pfhrp2 and pfhrp3 genes deletion in *Plasmodium falciparum* isolates and the implications for rapid diagnostic tests in Nigeria. *Acta Tropica*, *196*, 121–125. https://doi.org/10.1016/j.actatropica.2019.05.016

Gamboa, D., Ho, M. F., Bendezu, J., Torres, K., Chiodini, P. L., Barnwell, J. W., Incardona, S., Perkins, M., Bell, D., McCarthy, J., & 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). https://doi.org/10.1371/journal.pone.0008091

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M. S., Nene, V., Barrell, B. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature*, *419*(6906), 498–511. https://doi.org/10.1038/nature01097

Gary, R. E., Cannon, J. W., & Foster, W. A. (2009). Effect of sugar on male *Anopheles gambiae* mating performance, as modified by temperature, space, and body size. *Parasites and Vectors*, *2*(1), 1–13. https://doi.org/10.1186/1756-3305-2-19

Gatton, M. L., Ciketic, S., Barnwell, J. W., Cheng, Q., Chiodini, P. L., Incardona, S., Bell, D., Cunningham, J., & González, I. J. (2018). An assessment of false positive rates for malaria rapid diagnostic tests caused by non-plasmodium infectious agents and immunological factors. *PLoS ONE*, *13*(5), 1–9. https://doi.org/10.1371/journal.pone.0197395

Gendrot, M., Fawaz, R., Dormoi, J., Madamet, M., & Pradines, B. (2019). Genetic diversity and deletion of *Plasmodium falciparum* histidine-rich protein 2 and 3: a threat to diagnosis of *P. falciparum* malaria. *Clinical Microbiology and Infection*, *25*(5), 580–585. https://doi.org/10.1016/j.cmi.2018.09.009

Gerstl, S., Dunkley, S., Mukhtar, A., Smet, M. De, Baker, S., & Maikere, J. (2010). Assessment of two malaria rapid diagnostic tests in children under five years of age, with follow-up of false-positive pLDH test results, in a hyperendemic falciparum malaria area, Sierra Leone. *Malaria Journal*, *9*(28), 67–74.

Ghana Health Service (GHS). (2002). *Annual Report*.

Ghana Health Service (GHS). (2007). *The Health Sector in Ghana Facts and Figures 2007*.

Ghana Health Service (GHS). (2014a). *Guidelines for Case Management of Malaria in Ghana*.

Ghana Health Service (GHS). (2014b). *Guidelines for Case Management of Malaria in Ghana*.

Ghana Health Service (GHS). (2014c). *The Health Sector in Ghana Facts and Figures 2014*.

Ghana Health Service (GHS). (2018). *The Health Sector in Ghana Facts and Figures 2018*.

Ghana Statistical Service (GSS). (2014a). *District Analytical report of Tamale metropolis*.

Ghana Statistical Service (GSS). (2014b). *District Analytical report of West Gonja district*. https://www.statsghana.gov.gh/

Ghana Statistical Service (GSS). (2021). *Population of Regions and Districts*. https://www.statsghana.gov.gh/

Ghanchi, N. K., Qurashi, B., Raees, H., & Beg, M. A. (2021). Molecular surveillance of drug resistance: *Plasmodium falciparum* artemisinin resistance single nucleotide polymorphisms in Kelch protein propeller (K13) domain from Southern Pakistan. *Malaria Journal*, *20*(176), 1–6. https://doi.org/10.1186/s12936-021-03715-0

Gillet, P., Scheirlinck, A., Stokx, J., De Weggheleire, A., Chaúque, H. S., Canhanga, O. D., Tadeu, B. T., Mosse, C. D., Tiago, A., Mabunda, S., Bruggeman, C., Bottieau, E., & Jacobs, J. (2011). Prozone in malaria rapid diagnostics tests: How many cases are missed? *Malaria Journal*, *10*, 1–11. https://doi.org/10.1186/1475-2875-10-166

Ginsburg, H., & Deharo, E. (2011). A call for using natural compounds in the development of new antimalarial treatments- An introduction. *Malaria Journal*, *10*(SUPPL. 1). https://doi.org/10.1186/1475-2875-10-S1-S1

Grandesso, F., Nabasumba, C., Nyehangane, D., Page, A. L., Bastard, M., De Smet, M., Boum, Y., & Etard, J. F. (2016). Performance and time to become negative after treatment of three malaria rapid diagnostic tests in low and high malaria transmission settings. *Malaria Journal*, *15*(1), 1–12. https://doi.org/10.1186/s12936-016-1529-6

Grant, F. F. (2008). Synopsis of Causation Malaria. In *Ministry of Defence* (Vol. 1, Issue 9).

Grignard, L., Nolder, D., Sepúlveda, N., Berhane, A., Mihreteab, S., Kaaya, R., Phelan, J., Moser, K., van Schalkwyk, D. A., Campino, S., Parr, J. B., Juliano, J. J., Chiodini, P., Cunningham, J., Sutherland, C. J., Drakeley, C., & Beshir, K. B. (2020). A novel multiplex qPCR assay for detection of *Pfhrp2* and *P*fhrp3 deletions in polyclonal infections. *EBioMedicine*, *55*. https://doi.org/10.1016/j.ebiom.2020.102757

Guerra-Neira, A., Rubio, J. M., Royo, J. R., Ortega, J. C., Auñón, A. S., Diaz, P. B., & Llanes, A. B. (2006). *Plasmodium* diversity in non-malaria individuals from the Bioko Island in Equatorial Guinea (West Central-Africa). *International Journal of Health Geographics*, *5*(27), 1–8. https://doi.org/10.1186/1476-072X-5-27

Gupta, H., Matambisso, G., Galatas, B., Cisteró, P., Nhamussua, L., Simone, W., Cunningham, J., Rabinovich, N. R., Alonso, P., Saute, F., Aide, P., & Mayor, A. (2017). Molecular surveillance of *Pfhrp2* and *Pfhrp3* deletions in *Plasmodium falciparum* isolates from Mozambique. *Malaria Journal*, 1–7. https://doi.org/10.1186/s12936-017-2061-z

Haberichter, K. L., Johnson, P. C., Chittick, P. J., Millward, P., Robinson-Dunn, B., & Boyanton, B. L. (2017). The brief case: False-positive rapid malaria antigen test result in a returned traveler. *Journal of Clinical Microbiology*, *55*(8), 2294–2297. https://doi.org/10.1128/JCM.02347-16

Harhangi, B. S., Oostra, B. A., Heutink, P., Van Duijn, C. M., Hofman, A., & Breteler, M. M. B. (1999). N-acetyltransferase-2 polymorphism in Parkinson's disease: The Rotterdam study. *Journal of Neurology Neurosurgery and Psychiatry*, *67*(4), 518–520. https://doi.org/10.1136/jnnp.67.4.518

Herman, C., Huber, C. S., Jones, S., Steinhardt, L., Plucinski, M. M., Lemoine, J. F., Chang, M., Barnwell, J. W., Udhayakumar, V., & Rogier, E. (2019). Multiplex malaria antigen detection by bead-based assay and molecular confirmation by PCR

shows no evidence of *Pfhrp2* and *Pfhrp3* deletion in Haiti. *Malaria Journal*, *18*(1). https://doi.org/10.1186/s12936-019-3010-9

Hong, S. C. (2011). Malaria and economic productivity: A longitudinal analysis of the American case. *Journal of Economic History*, *71*(3), 654–671. https://doi.org/10.1017/S0022050711001872

Iriart, X., Menard, S., Chauvin, P., Mohamed, H. S., Charpentier, E., Mohamed, M. A., Berry, A., & Aboubaker, M. H. (2020). Misdiagnosis of imported Falciparum malaria from African areas due to an increased prevalence of *Pfhrp2/Pfhrp3* gene deletion: the Djibouti case. In *Emerging Microbes and Infections* (Vol. 9, Issue 1, pp. 1984–1987). Taylor and Francis Ltd. https://doi.org/10.1080/22221751.2020.1815590

Jensen, R. A., Adams, Y., & Hviid, L. (2019). Cerebral Plasmodium falciparum malaria: The role of PfEMP1 in its pathogenesis and immunity, and PfEMP1-based vaccines to prevent it. *Immunological Reviews*, *293*(1), 230–252. https://doi.org/10.1111/imr.12807

Kantele, A., & Jokiranta, T. S. (2011). Review of cases with the emerging fifth human malaria parasite, *Plasmodium knowlesi*. *Clinical Infectious Diseases*, *52*(11), 1356–1362. https://doi.org/10.1093/cid/cir180

Kavunga-Membo, H., Ilombe, G., Masumu, J., Matangila, J., Imponge, J., Manzambi, E., Wastenga, F., Ngoyi, D. M., Geetruyden, J. P. Van, & Muyembe, J. J. (2018). Molecular identification of *Plasmodium* species in symptomatic children of

Democratic Republic of Congo. *Malaria Journal*, *17*(334), 1–7. https://doi.org/10.1186/s12936-018-2480-5

Kihara, M., Carter, J. A., & Newton, Ch. R. J. C. (2006). The effect of Plasmodium falciparum on cognition: a systematic review. *Tropical Medicine and International Health*, *2*(4), 386–397. https://doi.org/10.1111/j.1365-3156.2006.01579.x

Kilejian, A. (1974). Histidine-rich polypeptide from malaria parasite, *Plasmodium lophurae*. *Journal of Biological Chemistry*, *249*(14), 4650–4655. https://doi.org/10.1016/S0021-9258(19)42468-X

Killeen, G. F., Masalu, J. P., Chinula, D., Fotakis, E. A., Kavishe, D. R., Malone, D., & Okumu, F. O. (2017). Control of malaria vector mosquitoes by insecticide-treated combinations of window screens and eave baffles. *Emerging Infectious Diseases*, *23*(5), 782–789. https://doi.org/10.3201/eid2305.160662

Kim, D., Fedak, K., & Kramer, R. (2012). Reduction of Malaria Prevalence by Indoor Residual Spraying: A Meta-Regression Analysis. *Am. J. Trop. Med. Hyg*, *87*(1), 117–124. https://doi.org/10.4269/ajtmh.2012.11-0620

King, M., George, A. E., Cisteró, P., Tarr-Attia, C. K., Arregui, B., Omeonga, S., Chen, H., Meyer García-Sípido, A., Sarukhan, A., Bassat, Q., Lansana, D. P., & Mayor, A. (2021). No evidence of false-negative *Plasmodium falciparum* rapid diagnostic results in Monrovia, Liberia. *Malaria Journal*, *20*(1), 1–10. https://doi.org/10.1186/s12936-021-03774-3

Kobayashi, T., Sikalima, J., Parr, J. B., Chaponda, M., Stevenson, J. C., Thuma, P. E., Mulenga, M., Meshnick, S. R., & Moss, W. J. (2019). The search for *Plasmodium falciparum* histidine-rich protein 2/3 deletions in Zambia and implications for plasmodium falciparum histidine-rich protein 2-based rapid diagnostic tests. *American Journal of Tropical Medicine and Hygiene*, *100*(4), 842–845. https://doi.org/10.4269/ajtmh.18-0859

Koita, O. A., Doumbo, O. K., Ouattara, A., Tall, L. K., Konaré, A., Diakité, M., Diallo, M., Sagara, I., Masinde, G. L., Doumbo, S. N., Dolo, A., Tounkara, A., Traoré, I., & Krogstad, D. J. (2012). False-negative rapid diagnostic tests for malaria and deletion of the histidine-rich repeat region of the hrp2 gene. *American Journal of Tropical Medicine and Hygiene*, *86*(2), 194–198. https://doi.org/10.4269/ajtmh.2012.10-0665

Kojom, L. P., & Singh, V. (2020). Prevalence of *Plasmodium falciparum* field isolates with deletions in histidine-rich protein 2 and 3 genes in context with sub-Saharan Africa and India: A systematic review and meta-analysis. *Malaria Journal*, *19*(1), 1–14. https://doi.org/10.1186/s12936-019-3090-6

Koram, K. A. (2003). *Mapping response of Plasmodium falciparum to chloroquine and other antimalarial drugs in Ghana*.

Koram, K. A., Abuaku, B., Duah, N., & Quashie, N. (2005). Comparative Efficacy of anti-malarial drugs including ACTs in the treatment of uncomplicated malaria in

young children under 5 in Ghana. *Acta Tropica*, *95*(October 2005), 194–203. https://doi.org/10.1016/j.actatropica.2005.06.018

Kozycki, C. T., Umulisa, N., Rulisa, S., Mwikarago, E. I., Musabyimana, J. P., Habimana, J. P., Karema, C., & Krogstad, D. J. (2017). False-negative malaria rapid diagnostic tests in Rwanda: impact of *Plasmodium falciparum* isolates lacking hrp2 and declining malaria transmission. *Malaria Journal*, *16*(1). https://doi.org/10.1186/s12936-017-1768-1

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Lee, J. H., Jang, J. W., Cho, C. H., Kim, J. Y., Han, E. T., Yun, S. G., & Lim, C. S. (2014). False-positive results for rapid diagnostic tests for malaria in patients with rheumatoid factor. *Journal of Clinical Microbiology*, *52*(10), 3784–3787. https://doi.org/10.1128/JCM.01797-14

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. *Journal of Clinical Microbiology*, *44*(8), 2773–2778. https://doi.org/10.1128/JCM.02557-05

Lee, N., Gatton, M. L., Pelecanos, A., Bubb, M., Gonzalez, I., Bell, D., Cheng, Q., & McCarthy, J. S. (2012). Identification of optimal epitopes for *Plasmodium*

falciparum rapid diagnostic tests that target histidine-rich proteins 2 and 3. *Journal of Clinical Microbiology*, *50*(4), 1397–1405. https://doi.org/10.1128/JCM.06533-11

Leigh, J. W., & Bryant, D. (2015). POPART: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, *6*(9), 1110–1116. https://doi.org/10.1111/2041-210X.12410

Li, B., Sun, Z., Li, X., Li, X., Wang, H., Chen, W., Chen, P., Qiao, M., & Mao, Y. (2017). Systematic review/Meta-analysis Performance of PfHRP2 versus pLDH antigen rapid diagnostic tests for the detection of *Plasmodium falciparum*: a systematic review and meta-analysis. *Systematic Review*, *13*(3), 541–549.

Li, N., Parker, D. M., Yang, Z., Fan, Q., Zhou, G., Ai, G., Duan, J., Lee, M. C., Yan, G., Matthews, S. A., Cui, L., & Wang, Y. (2013). Risk factors associated with slide positivity among febrile patients in a conflict zone of north-eastern Myanmar along the China-Myanmar border. *Malaria Journal*, *12*(1), 1–8. https://doi.org/10.1186/1475-2875-12-361

Luchavez, J., Baker, J., Alcantara, S., Belizario, V., Cheng, Q., Mccarthy, J. S., & Bell, D. (2011). Laboratory demonstration of a prozone-like effect in HRP2-detecting malaria rapid diagnostic tests: implications for clinical management. *Malaria Journal*.

Luchavez, J., Baker, J., Alcantara, S., Belizario, V., Cheng, Q., McCarthy, J. S., & Bell, D. (2011). Laboratory demonstration of a prozone-like effect in HRP2-detecting

malaria rapid diagnostic tests: Implications for clinical management. *Malaria Journal*, *10*, 1–7. https://doi.org/10.1186/1475-2875-10-286

Mahgoub, H., Gasim, G. I., Musa, I. R., & Adam, I. (2012). Severe *Plasmodium vivax* malaria among Sudanese children at New Halfa Hospital, Eastern Sudan. *Parasites and Vectors*, *5*(1), 2–5. https://doi.org/10.1186/1756-3305-5-154

Maltha, J., Gillet, P., & Jacobs, J. (2013). Malaria rapid diagnostic tests in travel medicine. *Clinical Microbiology and Infection*, *19*(5), 408–415. https://doi.org/10.1111/1469-0691.12152

Manske, M. (2006). *GENtle, a free multi-purpose molecular biology tool*.

Mba, C. J., & Aboh, I. K. (2004). Prevalence and Management of Malaria in Ghana: A Case Study of Volta Region.

Mbenda, H. G. N., & Das, A. (2014). Molecular evidence of *Plasmodium vivax* mono and mixed malaria parasite infections in Duffy-Negative native Cameroonians. *PLoS ONE*, *9*(8).

Ménard, D., Barnadas, C., Bouchier, C., Henry-halldin, C., & Gray, L. R. (2010). *Plasmodium vivax* clinical malaria is commonly observed in Duffy-negative Malagasy people. *PNAS*, *107*(13), 5967–5971. https://doi.org/10.1073/pnas.0912496107

Mendelson, M. (2013). Approach to the Patient with Malaria. *Travel Medicine: Third Edition*, 173–177. https://doi.org/10.1016/B978-1-4557-1076-8.00017-X

Mendes, C., Dias, F., Figueiredo, J., Mora, V. G., Cano, J., De, B., Benito, A., Berzosa, P., Arez, A. P., & Rosa, E. (2011). Duffy Negative Antigen Is No Longer a Barrier to P*lasmodium vivax*– Molecular Evidences from the African West Coast (Angola and Equatorial Guinea). *PLoS Neglected Tropical Diseases*, *5*(6), 2–7. https://doi.org/10.1371/journal.pntd.0001192

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. *Tropical Medicine and International Health*, *12*(2), 238–244.

Metoh, T. N., Chen, J. H., Gah, P. F., Zhou, X., & Somo, R. M. (2020). Genetic diversity of *Plasmodium falciparum* and genetic profile in children affected by uncomplicated malaria in Cameroon. *Malaria Journal*, *19*(115), 1–15. https://doi.org/10.1186/s12936-020-03161-4

Miller, R. H., Obuya, C. O., Wanja, E. W., Ogutu, B., Waitumbi, J., Luckhart, S., & Stewart, V. A. (2015). Characterization of *Plasmodium ovale curtisi* and *P. ovale wallikeri* in Western Kenya Utilizing a Novel Species-specific Real-time PCR Assay. *PLoS Neglected Tropical Diseases*, *9*(1), 1–19. https://doi.org/10.1371/journal.pntd.0003469

Ministry of Health. (2004). *Anti-malaria drug policy for Ghana*.

Moehrle, J. J. (2022). Development of New Strategies for Malaria Chemoprophylaxis: From Monoclonal Antibodies to Long-Acting Injectable Drugs. In *Tropical Medicine and Infectious Disease* (Vol. 7, Issue 58). MDPI. https://doi.org/10.3390/tropicalmed7040058

Moody, A. (2002). Rapid Diagnostic Tests for Malaria Parasites. *Clinical Microbiology Reviews*, *15*(1), 66–78. https://doi.org/10.1128/CMR.15.1.66

Morrow, R. (2007). *Epidemiology and Control of Malaria*.

Moser, K. A., Drábek, E. F., Dwivedi, A., Stucke, E. M., Crabtree, J., Dara, A., Shah, Z., Adams, M., Li, T., Rodrigues, P. T., Koren, S., Phillippy, A. M., Munro, J. B., Ouattara, A., Sparklin, B. C., Hotopp, J. C. D., Lyke, K. E., Sadzewicz, L., Tallon, L. J., Silva, J. C. (2020). Strains used in whole organism *Plasmodium falciparum* vaccine trials differ in genome structure, sequence, and immunogenic potential. *Genome Medicine*, *12*(6), 1–17.

Mouatcho, J. C., & Goldring, J. P. D. (2013). Malaria rapid diagnostic tests: challenges and prospects. *Medical Microbiology*, *62*, 1491–1505. https://doi.org/10.1099/jmm.0.052506-0

Moukah, M. O., Ba, O., Ba, H., Lemine, M., & Khairy, O. (2016). Malaria in three epidemiological strata in Mauritania. *Malaria Journal*, *15*(204), 1–14. https://doi.org/10.1186/s12936-016-1244-3

Murillo, S. C., Akinyi, O. S., Abdallah, J. F., Pava, Z., Dorado, E., Incardona, S., Huber, C. S., Macedo de Oliveira, A., Bell, D., Udhayakumar, V., & Barnwell, J. W. (2015). Deletion of *Plasmodium falciparum* Histidine-Rich Protein 2 (Pfhrp2) and Histidine-Rich Protein 3 (Pfhrp3) Genes in Colombian Parasites. *PloS One*, *10*(7), e0131576. https://doi.org/10.1371/journal.pone.0131576

Mze, N. P., Ahouidi, A. D., Diedhiou, C. K., Silai, R., Diallo, M., Ndiaye, D., Sembene, M., & Mboup, S. (2016). Distribution of *Plasmodium* species on the island of Grande Comore on the basis of DNA extracted from rapid diagnostic tests. *Parasite*, *23*(34). https://doi.org/10.1051/parasite/2016034

Nag, S., Kofoed, P. E., Ursing, J., Lemvigh, C. K., Allesøe, R. L., Rodrigues, A., Svendsen, C. A., Jensen, J. D., Alifrangis, M., Lund, O., & Aarestrup, F. M. (2018). Direct whole-genome sequencing of *Plasmodium falciparum* specimens from dried erythrocyte spots. *Malaria Journal*, *17*(1), 1–8. https://doi.org/10.1186/s12936-018-2232-6

Nderu, D., Kimani, F., Thiong'o, K., Karanja, E., Akinyi, M., Too, E., Chege, W., Nambati, E., Meyer, C. G., & Velavan, T. P. (2019). *Plasmodium falciparum* histidine-rich protein (PfHRP2 and 3) diversity in Western and Coastal Kenya. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-018-38175-1

Ngernna, S., Rachaphaew, N., Thammapalo, S., Prikchoo, P., Kaewnah, O., Manopwisedjaroen, K., Phumchuea, K., Suansomjit, C., Roobsoong, W., Sattabongkot, J., Cui, L., & Nguitragool, W. (2019). Case report: Case series of

human *Plasmodium knowlesi* infection on the Southern border of Thailand. *American Journal of Tropical Medicine and Hygiene*, *101*(6), 1397–1401. https://doi.org/10.4269/ajtmh.19-0063

Nyataya, J., Waitumbi, J., Mobegi, V. A., Noreddin, A., & El Zowalaty, M. E. (2020). *Plasmodium falciparum* Histidine-Rich Protein 2 and 3 Gene Deletions and Their Implications in Malaria Control. *Diseases*, *8*(2), 15. https://doi.org/10.3390/diseases8020015

Oguike, M. C., Betson, M., Burke, M., Nolder, D., Stothard, J. R., Kleinschmidt, I., Proietti, C., Bousema, T., Ndounga, M., Tanabe, K., Ntege, E., Culleton, R., & Sutherland, C. J. (2011). *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* circulate simultaneously in African communities. *International Journal for Parasitology*, *41*(6), 677–683. https://doi.org/10.1016/j.ijpara.2011.01.004

Okoth, S. A., Abdallah, J. F., Ceron, N., Adhin, M. R., Chandrose, J., Krishnalall, K., Huber, C. S., Goldman, I. F., Macedo de Oliveira, A., Barnwell, J. W., & Udhayakumar, V. (2015). Variation in *Plasmodium falciparum* histidine-rich protein 2 (*Pfhrp2*) and *Plasmodium falciparum* histidine-rich protein 3 (Pfhrp3) gene deletions in Guyana and Suriname. *PLoS ONE*, *10*(5).

Onguéné, A. P., Ntie-Kang, F., Likowo Lifongo, L., Claude Ndom, J., Sippl, W., & Meva, L. (2013). The potential of anti-malarial compounds derived from African medicinal plants. Part I: A pharmacological evaluation of alkaloids and terpenoids. *Malaria Journal*, *12*(1), 449. http://www.malariajournal.com/content/12/1/449

Orish, V. N., De-Gaulle, V. F., & Sanyaolu, A. O. (2018). Interpreting rapid diagnostic test (RDT) for *Plasmodium falciparum*. *BMC Research Notes*, *11*(1), 1–6. https://doi.org/10.1186/s13104-018-3967-4

Owusu, E. D. A., Brown, C. A., Grobusch, M. P., & Mens, P. (2017). Prevalence of Plasmodium falciparum and non-*P. falciparum* infections in a highland district in Ghana, and the influence of HIV and sickle cell disease. *Malaria Journal*, *16*(1), 10–17. https://doi.org/10.1186/s12936-017-1823-y

Oyola, S. O., Ariani, C. V., Hamilton, W. L., Kekre, M., Amenga-Etego, L. N., Ghansah, A., Rutledge, G. G., Redmond, S., Manske, M., Jyothi, D., Jacob, C. G., Otto, T. D., Rockett, K., Newbold, C. I., Berriman, M., & Kwiatkowski, D. P. (2016). Whole genome sequencing of *Plasmodium falciparum* from dried blood spots using selective whole genome amplification. *Malaria Journal*, *15*(1), 1–12. https://doi.org/10.1186/s12936-016-1641-7

Pain, A., Böhme, U., Berry, A. E., Mungall, K., Finn, R. D., Jackson, A. P., Mourier, T., Mistry, J., Pasini, E. M., Aslett, M. A., Balasubrammaniam, S., Borgwardt, K., Brooks, K., Carret, C., Carver, T. J., Cherevach, I., Chillingworth, T., Clark, T. G., Galinski, M. R., … Berriman, M. (2008). The genome of the simian and human malaria parasite *Plasmodium knowlesi*. *Nature*, *455*(7214), 799–803. https://doi.org/10.1038/nature07306

Parr, J. B., Verity, R., Doctor, S. M., Janko, M., Carey-ewend, K., Turman, B. J., Keeler, C., Slater, H. C., Whitesell, A. N., Mwandagalirwa, K., Ghani, A. C., Likwela, J.

L., Tshefu, A. K., Emch, M., Juliano, J. J., & Meshnick, S. R. (2017). Pfhrp2-Deleted *Plasmodium falciparum* parasites in the Democratic Republic of the Congo: A national cross- sectional survey. *Infectious Diseases*, *216*(1), 36–44. https://doi.org/10.1093/infdis/jiw538

Petersen, E. (2004). Malaria chemoprophylaxis: when should we use it and what are the options? *Expert Rev. Anti-Infect. Ther. 2*(1).

Phyo, P. A., & Nosten, F. (2018). The Artemisinin Resistance in Southeast Asia: An Imminent Global Threat to Malaria Elimination. In *Intech* (p. 25).

Plucinski, M. M., Candrinho, B., Dimene, M., Colborn, J., Lu, A., Nace, D., Zulliger, R., & Rogier, E. (2019). Assessing performance of HRP2 antigen detection for malaria diagnosis in Mozambique. *Journal of Clinical Microbiology*, *57*(9). https://doi.org/10.1128/JCM.00875-19

Plues, B., Tanser, F. C., Lengeler, C., & Sharp, B. L. (2010). Indoor residual spraying for preventing malaria (Review). In *Cochrane Database of Systematic Reviews* (Issue 4). https://doi.org/10.1002/14651858.CD006657.pub2.www.cochranelibrary.com

Poirier, P., Lang, C. D., Atchade, P. S., Lemoine, J. P., Louise, M., De, C., Abou, A., Pfaff, A. W., Brunet, J., Arnoux, L., Haar, E., Filisetti, D., Perrotey, S., Chabi, N. W., Akpovi, C. D., Anani, L., Bigot, A., Sanni, A., & Candolfi, E. (2016). The hide and seek of *Plasmodium vivax* in West Africa: report from a large-scale study in

Beninese asymptomatic subjects. *Malaria Journal*, *15*(570), 1–9. https://doi.org/10.1186/s12936-016-1620-z

Poser, C. M., & Bruyn, G. W. (1999). An illustrated history of malaria. *The Royal Society of Tropical Medicine and Hygiene*, *94*(2), 230.

Poti, K. E., Sullivan, D. J., Dondorp, A. M., & Woodrow, C. J. (2020). HRP2: Transforming malaria diagnosis, but with caveats. *Trends in Parasitology*, *36*(2), 112–126. https://doi.org/10.1016/j.pt.2019.12.004

Richie, T. L., & Saul, A. (2002). Progress and challenges for malaria vaccines. *Macmillan Journals Ltd*, *415*(February), 1–8.

Rios, L. M., & Roxanne, C. C. (2007). *Anopheles quadrimaculatus*. University of Florida. https://entnemdept.ufl.edu/creatures/aquatic/Anopheles_quadrimaculatus.htm

Robert, V., Le, G., Andrianaivolambo, L., & Randimby, F. M. (2006). Moderate transmission but high prevalence of malaria in Madagascar. *International Journal for Parasitology*, *36*(2006), 1273–1281. https://doi.org/10.1016/j.ijpara.2006.06.005

Roh, M. E., Oyet, C., Orikiriza, P., Wade, M., Kiwanuka, G. N., Mwanga-amumpaire, J., & Parikh, S. (2016). Asymptomatic *Plasmodium* Infections in Children in Low Malaria Transmission Setting, Southwestern Uganda. *Emerging Infectious Diseases*, *22*(8), 1–4.

Rozas, J., Ferrer-Mata, A., Sanchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sanchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution*, *34*(12), 3299–3302. https://doi.org/10.1093/molbev/msx248

Rubio, J. M., Benito, A., Roche, J., Berzosa, P. J., Garci´a, M. L., Garci´a, G., Mico´, M., Mico´, M., Edu´, M., & Alvar, J. (1999). Semi-Nested, Multiplex Polymerase Chain Reaction for Detection of Human Malaria Parasites and Evidence of *Plasmodium vivax* Infection in Equatorial Guinea. In *Am. J. Trop. Med. Hyg* (Vol. 60, Issue 2).

Russo, G., Faggioni, G., Paganotti, G. M., Bruna, G., Dongho, D., Pomponi, A., Santis, R. De, Tebano, G., Mbida, M., Sobze, M. S., Vullo, V., Rezza, G., & Lista, F. R. (2017). Molecular evidence of *P. vivax* infection in Duffy negative symptomatic individuals from Dschang, West Cameroon. *Malaria Journal*, 1–9. https://doi.org/10.1186/s12936-017-1722-2

Rutledge, G. G., Böhme, U., Sanders, M., Reid, A. J., Cotton, J. A., Maiga-Ascofare, O., Djimdé, A. A., Apinjoh, T. O., Amenga-Etego, L., Manske, M., Barnwell, J. W., Renaud, F., Ollomo, B., Prugnolle, F., Anstey, N. M., Auburn, S., Price, R. N., McCarthy, J. S., Kwiatkowski, D. P., Otto, T. D. (2017). *Plasmodium malariae* and *P. ovale* genomes provide insights into malaria parasite evolution. *Nature*, *542*(7639), 101–104. https://doi.org/10.1038/nature21038

Ryan, J. R., Stoute, J. A., Amon, J., Dunton, R. F., Mtalib, R., Koros, J., Owour, B., Luckhart, S., Wirtz, R. A., Barnwell, J. W., & Rosenberg, R. (2006). Evidence for transmission of *Plasmodium vivax* among a duffy antigen negative population in Western Kenya. *Am. J. Trop. Med. Hyg*, *75*(4), 575–581.

Salem, M., Ahmedou, O., Lekweiry, K. M., Deida, J. M., Emouh, A. O., Weddady, M. O., Ould, A., Salem, M., & Basco, L. K. (2015). Increasing Prevalence of Plasmodium vivax among Febrile Patients in Nouakchott, Mauritania. *Am. J. Trop. Med. Hyg*, *92*(3), 537–540. https://doi.org/10.4269/ajtmh.14-0243

Sato, S. (2021). *Plasmodium*- a brief introduction to the parasites causing human malaria and their basic biology. *Journal of Physiological Anthropology*, *9*(40:1), 1–13. https://doi.org/https://doi.org/10.1186/s40101-020-00251-9 (2021)

Sauerborn, R., Adams, A., & Hien, M. (1996). Household strategies to cope with the economic costs of illness. *Social Science and Medicine*, *43*(3), 291–301. https://doi.org/10.1016/0277-9536(95)00375-4

Schlabe, S., Reiter-Owona, I., Nordmann, T., Dolscheid-Pommerich, R., Tannich, E., Hoerauf, A., & Rockstroh, J. (2021). Rapid diagnostic test negative *Plasmodium falciparum* malaria in a traveler returning from Ethiopia. *Malaria Journal*, *20*(1), 1–7. https://doi.org/10.1186/s12936-021-03678-2

Schlagenhauf, P., & Petersen, E. (2008). Malaria chemoprophylaxis: Strategies for risk groups. In *Clinical Microbiology Reviews* (Vol. 21, Issue 3, pp. 466–472). https://doi.org/10.1128/CMR.00059-07

Sharp, B. L., Ridl, F. C., Govender, D., Kuklinski, J., & Kleinschmidt, I. (2007). Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea. *Malaria Journal*, *6*, 1–6. https://doi.org/10.1186/1475-2875-6-52

Shehu, D., Aminu, F. M., Shehu, D., & Mashi, J. A. (2021). Investigation of HRP2 Gene deletion in *Plasmodium falciparum* in Northwestern Nigeria. *Acta Biologica Marisiensis*, *4*(1), 10–20. https://doi.org/10.2478/abmj-2021-0002

Singh, B., Bobogare, A., Cox-Singh, J., Snounou, G., Shukri Abdullah, M., & Rahman, A. (1999). A Genus- and Species-specific nested Polymerase Chain Reaction malaria detection assay for epidemiologic studies. In *Am. J. Trop. Med. Hyg* (Vol. 60, Issue 4).

Singh, B., Sung, L. K., Matusop, A., Radhakrishnan, A., Shamsul, S. S. G., Cox-Singh, J., Thomas, A., & Conway, D. J. (2004). A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *The Lancet*, *363*(9414), 1017–1024. https://doi.org/10.1016/S0140-6736(04)15836-4

Snounou, G., Viriyakosol, S., Jarra, W., Thaithong, S., & Brown, K. N. (1993). Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Molecular and Biochemical Parasitology*, *58*(2), 283–292. https://doi.org/10.1016/0166-6851(93)90050-8

Srisutham, S., Suwannasin, K., Mathema, V. B., Sriprawat, K., Smithuis, F. M., Nosten, F., White, N. J., Dondorp, A. M., & Imwong, M. (2020). Utility of *Plasmodium falciparum* DNA from rapid diagnostic test kits for molecular analysis and whole genome amplification. *Malaria Journal*, *19*(1). https://doi.org/10.1186/s12936-020-03259-9

Stephens, J. W. W. (1922). A new malaria parasite of man. *Annals of Tropical Medicine and Parasitology*, *8*(1), 119–128. https://doi.org/10.1080/00034983.1914.11687646

Suuron, V. M., Mwanri, L., Tsourtos, G., & Owusu-Addo, E. (2020). An exploratory study of the acceptability of indoor residual spraying for malaria control in upper western Ghana. *BMC Public Health*, *20*(465), 1–11. https://doi.org/10.1186/s12889-020-08505-y

Symington, V. (2012). Malaria: A Global Challenge. In *Malaria: A Global Challenge*.

Target Malaria. (2020). *Anopheles gambiae s.l.: morphology, life-cycle, ecology* (Issue August).

Taylor, T., & Agbenyega, T. (2012). Infections of the blood and reticuloendothelial system. In *Hunter's Tropical Medicine and Emerging Infectious Disease* (Ninth Edit, pp. 695–717). https://doi.org/10.1016/B978-1-4160-4390-4.00096-5

Thomson, R., Beshir, K. B., Cunningham, J., Baiden, F., Bharmal, J., Bruxvoort, K. J., Maiteki-Sebuguzi, C., Owusu-Agyei, S., Staedke, S. G., & Hopkins, H. (2019).

Pfhrp2 and *Pfhrp3* Gene Deletions That Affect Malaria Rapid Diagnostic Tests for *Plasmodium falciparum*: Analysis of Archived Blood Samples From 3 African Countries. *The Journal of Infectious Diseases*, *220*(9), 1444–1452. https://doi.org/10.1093/infdis/jiz335

Tjitra, E., Anstey, N. M., Sugiarto, P., Warikar, N., Kenangalem, E., Karyana, M., Lampah, D. A., & Price, R. N. (2008). Multidrug-resistant *Plasmodium vivax* associated with severe and fatal malaria: A prospective study in Papua, Indonesia. *PLoS Medicine*, *5*(6), 0890–0899. https://doi.org/10.1371/journal.pmed.0050128

Tse, E. G., Korsik, M., & Todd, M. H. (2019). The past, present and future of anti-malarial medicines. In *Malaria Journal* (Vol. 18, Issue 93). BioMed Central Ltd. https://doi.org/10.1186/s12936-019-2724-z

Ugah, U. I., Alo, M. N., Owolabi, J. O., Divinegift, O., Nwali, O., Ekejindu, I. M., Ibeh, N., & Elom, M. O. (2017). Evaluation of the utility value of three diagnostic methods in the detection of malaria parasites in endemic area. *Malaria Journal*, *16*(189), 1–8. https://doi.org/10.1186/s12936-017-1838-4

United State Agency for International Development (USAID). (2012). Training Manual on Malaria Entomology for Entomology and Vector Control Technicians. *United States Agency for International Development Authors*, *54*(4), 186–188.

University of Thessaly, & European Union. (2013, October 27). *Information for healthcare professionals: epidemiology of malaria*. http://www.malwest.gr/en-us/malaria/informationforhealthcareprofessionals/plasmodiumlifecycle.aspx

Uwimana, A., Umulisa, N., Venkatesan, M., Svigel, S. S., Zhou, Z., Munyaneza, T., Habimana, R. M., Rucogoza, A., Moriarty, L. F., Sandford, R., Piercefield, E., Goldman, I., Ezema, B., Talundzic, E., Pacheco, M. A., Escalante, A. A., Ngamije, D., Mangala, J. L. N., Kabera, M., Lucchi, N. W. (2021). Association of *Plasmodium falciparum* Kelch13 R561H genotypes with delayed parasite clearance in Rwanda: an open-label, single-arm, multicenter, therapeutic efficacy study. *The Lancet Infectious Diseases*, *21*(8), 1120–1128. https://doi.org/10.1016/S1473-3099(21)00142-0

Van Loon, W., Oliveira, R., Bergmann, C., Habarugira, F., Ndoli, J., Sendegeya, A., Bayingana, C., & Mockenhaupt, F. P. (2022). In Vitro Confirmation of Artemisinin Resistance in *Plasmodium falciparum* from Patient Isolates, Southern Rwanda, 2019. *Emerging Infectious Diseases*, *28*(4), 852–855. https://doi.org/10.3201/eid2804.212269

Viana, G. M. R., Okoth, S. A., Silvia-Flannery, L., Barbosa, R. L., Macedo de Oliveira, A., Goldman, I. F., Morton, L. C., Huber, C., Annez, A., Machado, L. D. R., Camargo, Camargo. M. A., Negreiros do Valle, S. C., Povoa, M. M., Udhayakumar, V., & Barnwell, J. W. (2017). Histidine-rich protein 2 (pfhrp2) and pfhrp3 gene deletions in *Plasmodium falciparum* isolates from select sites in Brazil and Bolivia. *PLoS ONE*, *12*(3).

Warrell, D. A., & Gilles, H. M. (2017). Essential malariology. In *Taylor & Francis Group, LLC*. https://doi.org/10.1201/9780203756621

Wellems, T. E., & Howard, R. J. (1986). Homologous genes encode two distinct histidine-rich proteins in a cloned isolate of *Plasmodium falciparum*. *Genetics*, *83*(August), 6065–6069.

White, N. J. (2004). Antimalarial drug resistance. In *Journal of Clinical Investigation* (Vol. 113, Issue 8, pp. 1084–1092). The American Society for Clinical Investigation. https://doi.org/10.1172/JCI21682

Willie, N. (2018). *Plasmodium falciparum histidine- rich protein 2 gene variation and malaria detection in Madagascar and Papua New Guinea*. Case Western Reserve University.

Win, T. T., Jalloh, A., Tantular, I. S., Tsuboi, T., Ferreira, M. U., Kimura, M., & Kawamoto, F. (2004). Molecular analysis of *Plasmodium ovale* Variants. *Emerging Infectious Diseases*, *10*(7), 1235–1240.

World Health Organization (WHO). (2003). Malaria Rapid Diagnosis: Making it Work. In *Meeting report* (Vol. 05, Issue January). https://apps.who.int/iris/handle/10665/208030

World Health Organization (WHO). (2008). *World Health Report 2008*. https://www.who.int

World Health Organization (WHO). (2009). *Malaria case management: operations manual*.

https://www.who.int/malaria/publications/atoz/malaria_case_management_operations_manual.pdf

World Health Organization (WHO). (2010a). *Guidelines for the treatment of malaria*.

World Health Organization (WHO). (2010b). *World Malaria Report 2010*. https://www.who.int

World Health Organization (WHO). (2011). *WHO Global Malaria Programme: Good practices for selecting and procuring rapid diagnostic tests for malaria WHO Global Malaria Programme Good practices for selecting and procuring rapid diagnostic tests for malaria rapid diagnostic tests for malaria rapid*. https://www.who.int

World Health Organization (WHO). (2012). *T3: Scaling up diagnostic testing, treatment and surveillance for malaria*. www.who.int

World Health Organization (WHO). (2014). *Severe Malaria: Vol. 19 Suppl 1*. https://doi.org/10.1111/tmi.12313_2

World Health Organization (WHO). (2015a). *An operation manual for indoor residual spraying (IRS) for malaria transmission control and elimination*. https://www.who.int

World Health Organization (WHO). (2015b). *Global technical strategy for malaria 2016–2030*. https://www.who.int

World Health Organization (WHO). (2015c). *Guidelines for the treatment of malaria* (Vol. 35, Issue 4). https://doi.org/10.1046/j.1472-765X.2002.01179.x

World Health Organization (WHO). (2015d). *Malaria microscopy: quality assurance manual*.

World Health Organization (WHO). (2015e). *World Malaria Report 2015*. https://www.who.int

World Health Organization (WHO). (2016a). *Plasmodium knowlesi* current status and the request for review by an Evidence Review Group. In *Malaria, Other Vectorborne and Parasitic Disease (MVP)* (Issue September).

World Health Organization (WHO). (2016b). *Recommended selection criteria for procurement of malaria rapid diagnostic tests* (Issue March).

World Health Organization (WHO). (2017). *Expert consultation on Plasmodium knowlesi to guide malaria elimination strategies* (Issue 03). https://www.who.int

World Health Organization (WHO). (2018a). *Recommended selection criteria for procurement of malaria rapid diagnostic tests* (Issue December).

World Health Organization (WHO). (2018b). *World Malaria Report 2018*. World Health Organization. https://www.who.int

World Health Organization (WHO). (2019a). *A heavy burden: The indirect cost of illness in Africa*. https://www.who.int

World Health Organization (WHO). (2019b). *False-negative RDT results and P. falciparum histidine-rich protein 2/3 gene deletions* (Vol. 2, Issue 1). https://www.who.int

World Health Organization (WHO). (2019c). *World Malaria Report 2019*. World Health Organization. https://www.who.int

World Health Organization (WHO). (2020a). Malaria eradication: benefits, future scenarios and feasibility. In *Strategic Advisory Group on Malaria Eradication*. https://www.who.int/publications-detail/strategic-advisory-group-malaria-eradication-executive-summary

World Health Organization (WHO). (2020b). *Report on antimalarial drug efficacy, resistance and response*.

World Health Organization (WHO). (2020c). *Surveillance template protocol for pfhrp2/pfhrp3 gene deletions*. https://www.who.int

World Health Organization (WHO). (2020d). *World malaria report 2020: 20 years of global progress and challenges*. https://www.who.int

World Health Organization (WHO). (2021). *World Malaria Report 2021*. https://www.who.int

World Health Organization (WHO). (2022). *WHO Guidelines for Malaria*.

Wurtz, N., Fall, B., Bui, K., Pascual, A., Fall, M., Camara, C., Diatta, B., Fall, K. B., Mbaye, P. S., Diémé, Y., Bercion, R., Wade, B., Briolant, S., & Pradines, B. (2013). *Pfhrp2* and *Pfhrp3* polymorphisms in *Plasmodium falciparum* isolates from Dakar, Senegal: Impact on rapid malaria diagnostic tests. *Malaria Journal*, *12*(1), 1–8. https://doi.org/10.1186/1475-2875-12-34

Wurtz, N., Lekweiry, K. M., Bogreau, H., Pradines, B., Rogier, C., Ould, A., Salem, M., Hafid, J. E., Salem, M., Ahmedou, O., Trape, J., Basco, L. K., & Briolant, S. (2011). Vivax malaria in Mauritania includes infection of a Duffy-negative individual. *Malaria Journal*, *10*(336),1–8.

Zekar, L., & Sharman, T. (2021). Plasmodium falciparum malaria. *StatPearls Publishing*, *162*(4), 123–142.

APPENDICES

Appendix I: PeqSTAR 96X Universal thermal cycler (peQlab, VWR)

Appendix II: MicroDOC Gel Documentation System (Cleaver Scientific Ltd., UK)

Appendix III: Ethical clearance/approval by the SMHS_SAHS joint Institutional Review Board of the University for Development Studies

UNIVERSITY FOR DEVELOPMENT STUDIES

SCHOOL OF MEDICINE AND HEALTH SCIENCES AND SCHOOL OF ALLIED HEALTH SCIENCES JOINT INSTITUTIONAL REVIEW BOARD (SMHS-SAHS JIRB)

Tel:0372-022078/026633
Email: parmah@uds.edu.gh

Our Ref: JIRB280720
Your Ref:

P.O. Box 1883
Tamale, Ghana

July 28, 2020

(Office of the Chairman)

Dr. Cletus Adiyaga Wezena
Department of Microbiology,
Faculty of Biosciences,
University for Development Studies,
P O Box TL 1883
Tamale

ETHICS APPROVAL CERTIFICATE ID: SMSAHS/JIRB/0010

DATE APPROVED: July 21, 2020 **EXPIRATION DATE: June 20, 2021**

Dear Dr. Wezena,

Approval of protocol titled ***"Comparative analysis of Pfhrp2/3-dependent RDT and Pf-based PCR for detecting plasmodium falciparum infection in microscopy confirmed malaria positive blood samples from northern Ghana"***

I write to inform you that the SMHS_SAHS Joint IRB has undertaken an expedited review of the above-named protocol during its Board meeting held on May 20, 2019.

The documents that were reviewed and approved are as follows;

- Study protocol version 1.0
- Consent form – English Version 1.0
- Curriculum Vitae of Investigators

The Board also made a number of recommendations for your consideration. Please note that any further amendment to this approved protocol must receive ethical clearance from the IRB before its implementation. Should you require a renewal of your approval, a report should be submitted two (2) months before the expiration date.

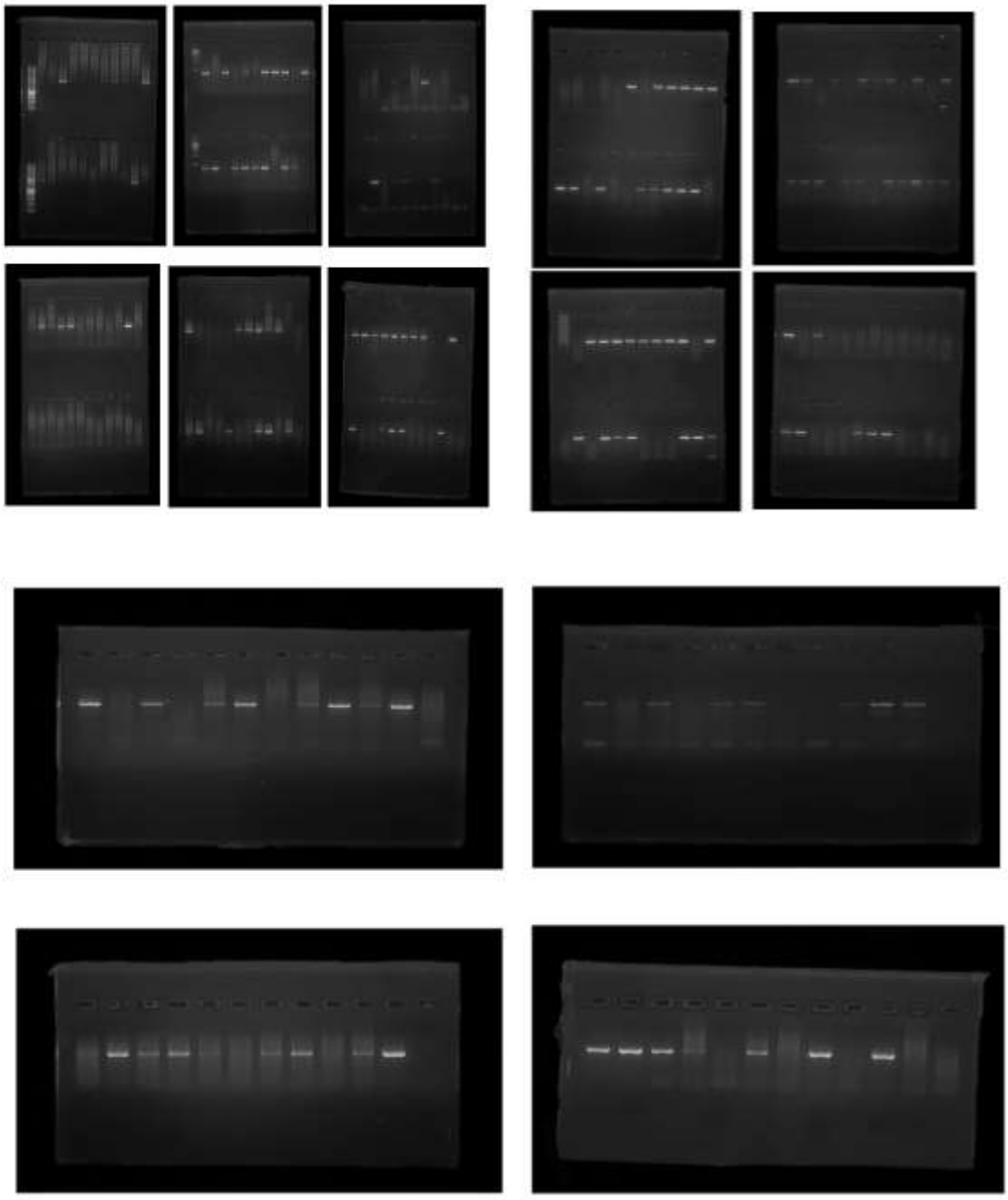
The Board wishes you the best in this study.

Sincerely,

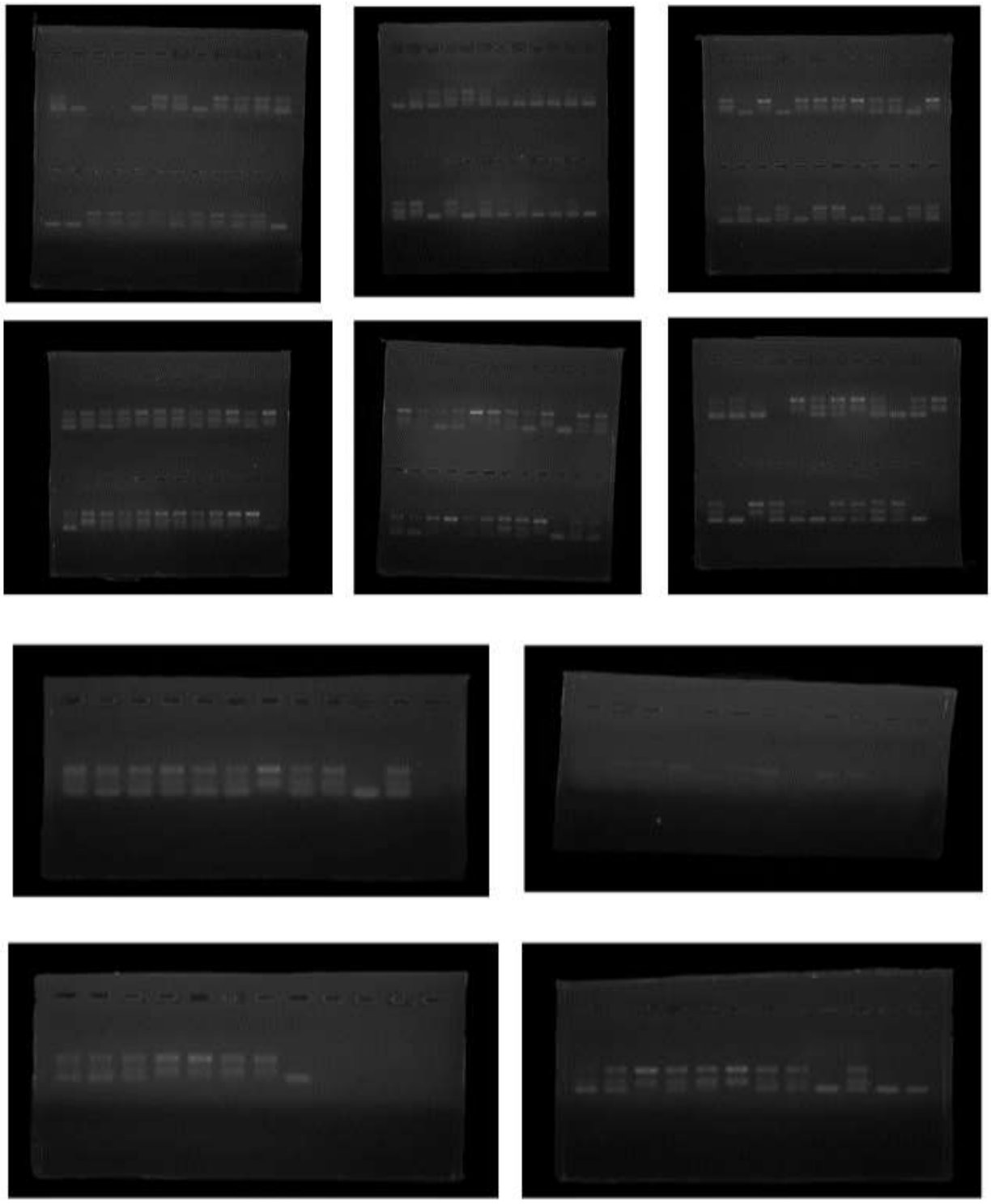
Prof. Paul Armah Aryee
(Chairman)

Prof. Gideon Kofi Helegbe
(Administrator)

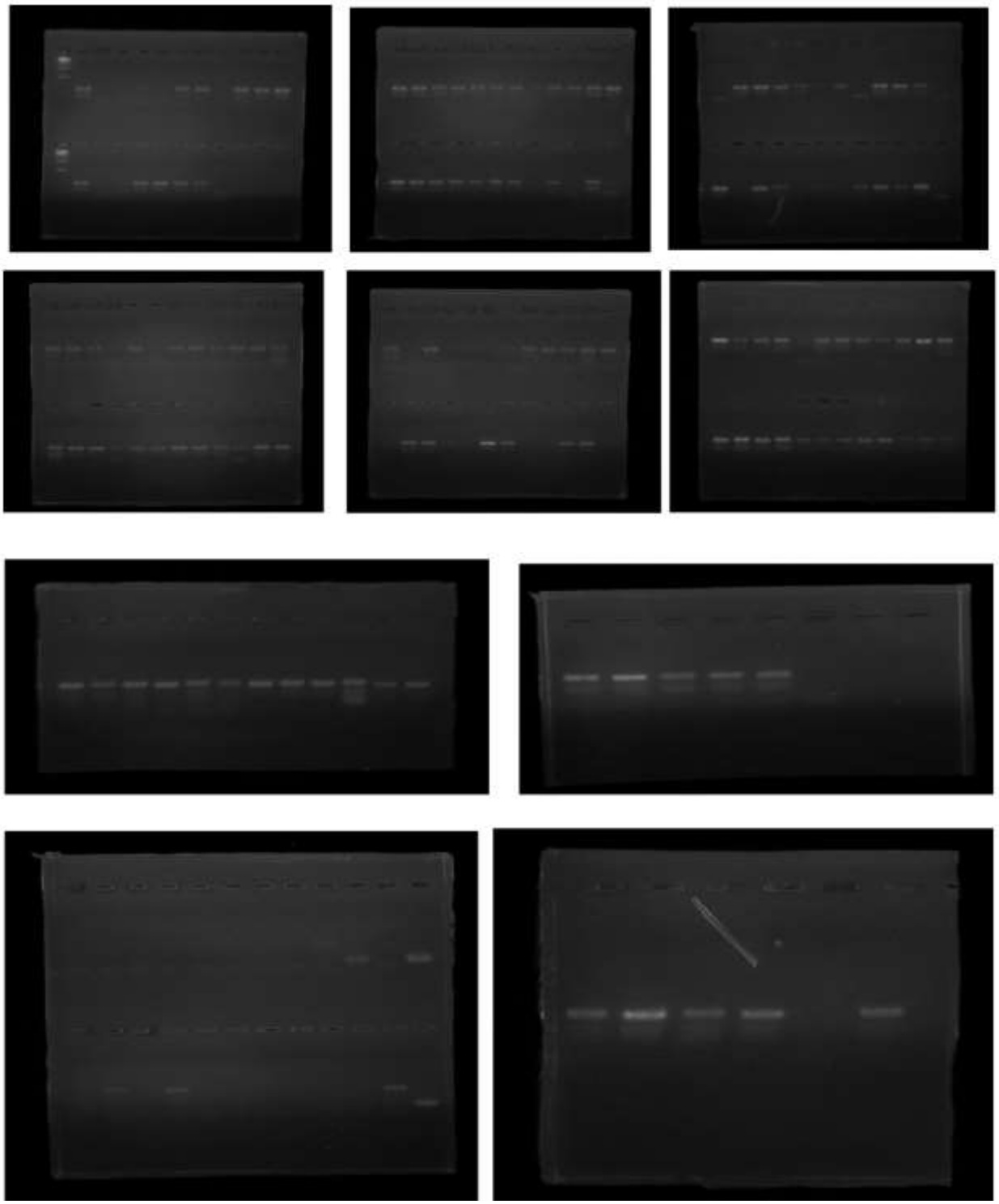
Appendix IV: Agarose gel (2 %, w/v) electrophoresis photos of 18S rRNA amplicons of *Plasmodium falciparum* (~395 bp).



Appendix V: Agarose gel (2.0 % w/v) electrophoresis photo of Exon1−2 *Pfhrp2* amplicons of *P. falciparum*



Appendix VI: Agarose gel (2.0 %, w/v) electrophoresis photos of Exon 1–2 *Pfhrp3* amplicons of *Plasmodium falciparum* (~228 bp)



Appendix VII: Part of results presented and published in the Book of Abstracts of the 14th Annual Interdisciplinary Conference, UDS

HISTIDINE-RICH PROTEIN 2/3 DEPENDENT RDT VS PCR-BASED ASSAY FOR DIAGNOSIS OF MALARIA IN HUMANS IN TAMALE, GHANA

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ABSTRACT

Malaria is caused by *Plasmodium* species and transmitted by female Anopheles mosquitoes. In humans, five species have been identified to cause infection. Though microscopy is the gold-standard for the diagnosis of malaria, RDT, and PCR assays are also commonly used. RDTs are based on the use of monoclonal antibodies to detect the antigenic proteins of *Plasmodium falciparum Pfhrp2/3* genes in human blood. Malaria RDTs are widely used in Ghana as a convenient alternative to microscopy. Our study investigated reliability of RDTs for the diagnosis of malaria in the Northern Region of Ghana. Blood samples from 84 malaria patients diagnosed by microscopy from Tamale Teaching Hospital were tested by

RDT and PCR using whole blood and extracted protozoan DNA, respectively. The PCR was conducted using a semi-nested method that detected the 16s rRNA gene. On a whole, RDT detected 49/84 (58%) samples to be positive for malaria while PCR revealed 47/84(56%) to be positive for *P. falciparum* infection. Out of the 47 samples that tested *P. falciparum* positive by PCR, 7(14.9%) tested negative by RDT. Also, 9/37 (24.3%) of the samples negative for *P. falciparum* by PCR tested positive for RDT. The 37 malaria positive blood samples that produced no positive results by PCR where *P. falciparum* specific primers were used could be as a result of other *Plasmodium* species that cause malaria in humans. Our results raise questions about the reliability of *P. falciparum* based RDTs used in Ghana but further validations are required for good appraisal of the challenge.

Appendix VIII: Part of results presented and published in the Book of Abstracts of the 1st Annual Graduate School Conference, UDS

PLASMODIUM FALCIPARUM HISTIDINE-RICH PROTEIN 2/3 DELETIONS IN SYMPTOMATIC MALARIA PATIENTS IN TAMALE METROPOLIS, GHANA

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Human malaria is caused by five *Plasmodium* species that are transmitted by the female *Anopheles* mosquitoes. PCR assays, RDT and microscopy are malaria diagnostic approaches with the latter deemed the gold standard. RDTs is a more convenient alternative that works on the principle of monoclonal antibodies detecting antigenic proteins coded by *Plasmodium* spp. genes such as the *Pfhrp2* gene in human blood. This study sought to determine the prevalence of *Pfhrp2/3* gene deletions in the Tamale Metropolis of Ghana in order to evaluate the reliability of *P. falciparum*-based RDTs in the diagnosis of malaria in the region. Blood samples from 84 malaria patients diagnosed by

microscopy from the Tamale Teaching Hospital were tested for *P. falciparum* infection by *P. falciparum Pfhrp*2-based RDT and PCR using whole blood and extracted protozoan DNA, respectively. Semi-nested PCR was conducted to detect the 18s rRNA gene of *P. falciparum* and further analysed for the presence or absence of the *Pfhrp2/3* gene. Forty-seven (47, 55.96%) samples were *P. falciparum* positive by 18s rRNA PCR out of which seven (14.89%) showed no product for both *Pfhrp*2&3 genes whereas one (2.13%) of the samples, showed product for *Pfhrp*3 but not *Pfhrp*2 gene. The seven samples that were negative for both genes by PCR were also negative by *hrp2*-dependent RDT indicating a false RDT negative malaria diagnosis. RDT was positive for the single sample that was positive for *Pfhrp*3 but not *Pfhrp*2, the primary target of the RDT. This confirms earlier reports of cross-reactivity between *Pfhrp*2-specific antibodies with *Pfhrp*3 antigen due to the sequence similarity between the two proteins. Our study revealed evidence of *Pfhrp2/3* deletion in *P. falciparum* causing malaria in the Tamale Metropolis. Further investigations are needed to understand the extent, and impact of *Pfhrp*2/3 mutations in the region.