

UNIVERSITY OF CAPE COAST

IDENTIFICATION OF *PLASMODIUM FALCIPARUM* HISTIDINE-RICH
PROTEIN II AND III (*PFHRP2/3*) GENE DELETIONS IN TWO COMMUNITIES
IN SOUTHERN GHANA: IMPLICATIONS ON RAPID DIAGNOSTIC TESTS

BY

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Thesis submitted to the Department of Molecular Biology and Biotechnology of the
School of Biological Sciences, College of Agricultural and Natural Sciences,
University of Cape Coast, in partial fulfillment of the requirements for the award of
Master of Philosophy degree in Molecular Biology and Biotechnology

JUNE 2018

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DIAGNOSTIC TESTS**

BY

SAMUEL BADU NYARKO (BSc)

JUNE 2018

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature:..... Date:.....

Name: Samuel Badu Nyarko

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Main Supervisor's Signature: Date:.....

Name: Dr. Daniel K.A. Asante

Supervisor's Signature:..... Date:.....

Name: Dr. Linda Eva Amoah

ABSTRACT

Malaria Rapid Diagnostic Tests (RDT's) have improved malaria diagnosis in highly endemic rural settings. However, the increasingly high false negative rates of *Plasmodium falciparum* histidine-rich protein II (PfHRP2) based RDT kits (PfHRP2-RDT) is a major obstacle to the rapid and reliable diagnosis of malaria. This study was aimed at determining the rate of false negative RDT as well as the prevalence of *P. falciparum* parasites with *pfhrp2* deletions in selected communities in the Southern Ghana. Whole blood was collected from volunteers living in Obom (high transmission) and Asutsuare (low transmission) and separated into plasma and cell pellet. Genomic DNA was extracted from 310 cell pellets from both sites using the ZymoDNA Kit[®]. Species-specific *18srRNA* PCR was used to identify *P. falciparum* positive samples. *Pfmsp2* and *glurp* genotyping was used to determine recrudescence or new infection. Good quality DNA samples were then subjected to *pfhrp2* exon 1 and 2 PCR as well as *pfhrp3* exon 2 PCR. PfHRP2 antigen level was determined using a *pfhrp2* Malaria Ag CELISA kit. Microscopy estimation of malaria parasites were 3.3 % of samples from Asutsuare against 39.8 % of Obom samples. The RDTs had 1.7 % of samples from Asutsuare while Obom had 53.4 %. Using *18srRNA* PCR for *P. falciparum* speciation, 59.1 % of the Asutsuare samples tested positive for the malaria parasite whereas 65.8 % of the Obom samples tested positive. *Plasmodium falciparum* parasites with deletions of both *pfhrp2* and *pfhrp3* gene were 1.7 % in Obom and 4.8 % in Asutsuare. An R-square value of 0.997 and 0.994 were obtained for Obom and Asutsuare Regression Analysis of ELISA respectively. Deletions of *pfhrp2* and *pfhrp3* genes were identified in the two study sites and there were higher quantities of PfHRP2 antigens in Obom than in Asutsuare.

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DEDICATION

To my family, my wife Vashty and my children, Uel and Hadassah

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LIST OF ABBREVIATIONS

PfHRP2	<i>P.falciparum</i> histidine rich protein II antigen
PfHRP3	<i>P.falciparum</i> histidine rich protein III antigen
<i>pfhrp2</i>	<i>P.falciparum</i> histidine rich protein II gene
<i>pfhrp3</i>	<i>P.falciparum</i> histidine rich protein III gene
<i>18srRNA</i>	18s ribosomal ribonucleic acid
CDC	Centre for Disease Control and Prevention
FIND	Foundation for Innovative New Diagnostics
GHS	Ghana Health Service
RDT	Rapid diagnostic test
WHO	World Health Organization
MOH	Ministry of Health
MSP2	Merozoite surface protein 2 (Antigene)
<i>mSP2</i>	Merozoite surface protein 2 (gene)
WHO	World Health Organization
bp	Base Pairs
CDC	Centre for Disease Control
CM	Cerebral Malaria
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphates
ELISA	Enzyme Linked Immunosorbent Assay
<i>Glurp</i>	Glutamate-Rich Protein

Hb	Haemoglobin
iRBC	Infected Red Blood Cells
ITNs	Insecticides Treated Nets
NMIMR	Noguchi Memorial Institute for Medical Research
OPD	Out Patient Department
PBS	Phosphate Buffer Saline
RBC	Red Blood Cells
SM	Severe Malaria
TAE	Tris-Acetate EDTA
UM	Uncomplicated Malaria
WBC	White Blood Cells

CHAPTER ONE

INTRODUCTION

Background

Malaria is a disease of the poor, which significantly affect endemic countries' economies (Kumar *et al.*, 2013). For complete eradication of malaria to be achieved, all forms of the disease need to be diagnosed and treated. Malaria diagnosis is expensive when specificity and accuracy is desired for prompt treatment. Microscopy has been the gold standard for malaria diagnosis for many years following various staining procedures (WHO, 2010). There are other tests which have also been used in the diagnosis for the infection. Recently, prompt treatments of malaria have largely been due to the introduction of Rapid Diagnostic Tests (RDTs). The use of RDT has been a very essential component of malaria diagnosis (Hanscheid, 1999; Kumar *et al.*, 2013; Wongsrichanalai, Barcus, Muth, Sutamihardja & Wernsdorfer, 1999; Wongsrichanalai, 2001), and has improved diagnosis in endemic and resource constraint settings (Makler, Palmer & Ager, 1998). In areas where there are no microscopists, RDTs are the major diagnostic tool for malaria infection diagnosis.

The malaria RDT principle is based on antigens or antibodies for detection. The most widely used diagnostic RDT for *Plasmodium falciparum* is based on detection of the histidine rich protein II (HRP2) antigen. *Plasmodium falciparum* has several *hrp* genes but only *hrp2* is used in RDTs. The *P. falciparum hrp (pfhrp2)* gene is heat stable, abundant in the host's blood and can

persist for 28 days or more after parasite clearance (Iqbal, Siddique, Jameel & Hira, 2004; Kumar *et al.*, 2012). The *P. falciparum* histidine-rich protein III (PfHRP3) antigens are known to have similar structure as the *P. falciparum* histidine-rich protein II (PfHRP2) antigens. Thus, they are recognised by the PfHRP2 antibodies. The PfHRP2 based RDT kits have the highest detection rate (World Health Organization-Foundation for Innovative New Diagnostics, 2015), but are also known to give inaccurate results with false positives and false negatives (Gamboa *et al.*, 2010). One main demerit in malaria RDT diagnosis without the confirmation of microscopy and its related parasite density is false negative results. This may be due to low parasite densities or *P. falciparum* histidine-rich protein II (*pfhrp2*) gene deletions which have not been compensated for by the *P. falciparum* histidine-rich protein III (*pfhrp3*) gene (Bartoloni & Zammarchi, 2012). Adaptation of the *Plasmodium* parasite to fit in the constant environmental changes has led to certain *P. falciparum* strains having their entire *pfhrp2* and *pfhrp3* gene deleted (Cheng *et al.*, 2014) or others having varying amounts of PfHRP2 antigen (Houze, Hubert, Le Pessec & Clain, 2011; Ho *et al.*, 2014)). This, affects the test accuracy (Baiden *et al.*, 2014; Houze, Boly, Le Bras, Deloron & Faucher, 2009; Kattenberg *et al.*, 2012; World Health Organization, 2015; Wurtz *et al.*, 2013).

Current trends in the use of RDT has resulted in significant improvements in infection rate reporting from different countries in Africa. However, to date, a number of all these countries, including Democratic Republic of Congo, Kenya,

Mali, Senegal and Zambia have reported *pfhrp2/3* gene deletions (Deme *et al.*, 2014; Kabayinze *et al.*, 2008; Parr *et al.*, 2016; Wurtz *et al.*, 2013).

The demand for malaria RDT kits in malaria diagnosis have increased since the WHO recommended its usage in 2009 (WHO, 2015). Presently, most National Malaria Control Programs (NMCPs) in endemic countries are using RDTs as the initial step of disease diagnosis. To monitor the use of RDTs, WHO instituted the Foundation for Innovative New Diagnostics (FIND) for quality assurance of RDT, which includes Lot Testing (WHO-FIND, 2015). This has helped in taking out most of the unapproved RDTs from the health system (Cunningham, 2013). There are many different tests which can be used for diagnosis of malaria infection aside RDTs. However, improvement of RDTs is very essential for clinical diagnosis in the communities where there are no microscopists and good equipment for infection diagnosis.

The basis of any disease control scheme for community and individual level is the availability of suitable diagnostic tools, which are highly sensitive (200 p/μL) and 99 % specific (WHO, 2010). Without these, interventions put in place cannot be tracked and suitable treatment not provided. PfHRP2 RDTs have been used in recent times as the most readily available and fast diagnostic method for malaria infections (Cohen, Dupas & Schaner, 2015; Baker *et al.*, 2010). However, their false negative and positive rates have given much room for criticisms (Kumar *et al.*, 2013). It has also brought about treatment burdens (Cohen, *et al.*, 2015), since most false positives may be given treatment for malaria where there is actually no infection. This can cause hepatotoxicity leading

to liver damage from drug burden. False negatives also bring about no treatments of the disease and so persons living with the infection become reservoirs for the spread of the parasite as well as give the parasite more time for building drug resistance. This could be the reason why deaths of malaria are still recorded in this age when Artemisinin-based Combination Therapies (ACTs) have proven to be more effective for the treatment of malaria. This can cause low productivity and thus affect economies where *pfhrp2/pfhrp3* deletions are higher.

Low transmission areas have very few malaria cases as well as more clonal parasite infections and so other diseases which present similar symptoms are looked at and treated when PfHRP2 RDTs are negative for individuals living in the area. However, the results may be false negative which may be due to *pfhrp2/pfhrp3* gene deletions or low antigen levels of the PfHRP2 (Gamboa *et al.*, 2010). As a result, studies on gene deletions in low transmission areas are necessitated. On the other hand, high transmission areas were often seen as malaria prone and thus individuals presenting symptoms of malaria were still treated for malaria even when there were no parasites in the body (WHO, 2010). However, the current standard treatment guidelines require treatment only after individuals have tested positive for malaria RDT or microscopy (WHO, 2017).

In rural health facilities, malaria is often ruled out after RDT is tested negative, however, the patient may still be harbouring the parasites in his/her body. A study in Uganda determined that while 73% of febrile patients received antimalarials, only 35% had positive RDT results, and overall appropriate treatment was only 34% (Mbonye, Lal, Cundill, Hansen, Clarke & Magnussen,

2013). This may be due to gene deletions and false positives which made the RDT unable to capture the *P. falciparum* infection at the time as well as antigen persistence in the blood. These *pfhrp2/pfhrp3* deletants should be extensively studied in low transmission and high transmission areas to be able to find appropriate diagnostic method for high and low transmissions alike.

Statement of the Problem

Misdiagnosis of malaria due to false negatives and positives of RDT results is a menace to clinical patients and affected the setting of prevalence used for policy making and policy implementation. Although *PfHRP2* RDTs are cheaper, faster and easier to use compared to molecular tests and ELISA, they cannot be relied on to set the community prevalence of malaria (Laban *et al.*, 2015).

There is very little information about the extent of *pfhrp2/pfhrp3* deletions among *P. falciparum* isolates in Ghana, although there is a global increase in the prevalence of *P. falciparum* parasites with deletions in the *pfhrp2* gene. Due to the implications of the use of RDTs in Ghana for case managements and malaria elimination programmes, it is highly important to use reliable methods to determine the present prevalence of *pfhrp2* negative parasites in Southern Ghana.

Aim of the Study

To determine the presence of *P. falciparum* parasites with *pfhrp2* and *pfhrp3* genes deletions in Obom (high transmission area) and Asutsuare (low transmission area) in Southern Ghana.

Specific Objectives

1. To compare the clinical efficiency of *18s rRNA* species specific PCR with RDT and microscopy in *P. falciparum* infections in Obom and Asutsuare.
2. To identify and confirm the existence of *pfhrp2* and *pfhrp3* deletant *P. falciparum* isolates circulating in the two study sites.
3. To compare parasite positivity using PfHRP2 antigen levels in the plasma and PfHRP2 RDT.

Hypothesis of the Study

1. Clinical efficiency of microscopy and RDTs are very low in determining the prevalence of malaria in low malaria areas.
2. Low transmissions areas have few gene deletions while high transmission areas will have a higher number of *pfhrp2/pfhrp3* deletions due to higher diversity that may exists in the area.
3. Lower PfHRP2 concentrations may cause false negative RDT results due to low parasite density. Hence parasite positivity for RDT may be reduced though the parasite may be present in the sample.

Significance of the Study

RDTs have been used in health surveys and epidemiological studies for most diseases in recent years (Gitonga *et al.*, 2012; Plucinski *et al.*, 2017; Donald *et al.*, 2016). However, RDTs have limitations of producing false negative and false positive results and thus false information. The PfHRP2 RDT may provide a false

estimate of the prevalence of *P. falciparum* infection in a community as such RDT results should be confirmed by PCR if available or by microscopy before the prevalence of the area is established for policy making.

Only a few parasites with *pfhrp2* gene deletions have been found in areas of low transmission (Watson *et al.*, 2017). This study sets out to determine *pfhrp2* gene deletions existing in Asutsuare, a low transmission area of Ghana as well as determine the extent to which *pfhrp2* deletant parasites exist in Obom, a high transmission area.

There are inconsistencies in reporting parasite prevalence based on microscopy and RDT data provided by the National Malaria Control Programmes to the WHO. This has led to the WHO reporting a zero prevalence of *Plasmodium malariae* and *Plasmodium ovale* in Ghana for the past three consecutive years (WHO, 2015; WHO, 2016; WHO, 2017), but this has been proven to be false when molecular based assays were used to determine the prevalence of these other *Plasmodium* species (Owusu, Brown, Grobusch & Mens, 2018). Owusu *et al* (2018) reported an 18 % prevalence of *P. malariae* among 142 samples which were used for an HIV and sickle cell study. This highlights the need for additional specific and sensitive molecular methods including conventional PCR and quantitative PCR which should be used for disease monitoring and case management in malaria elimination reports.

Limitations

The use of qPCR as a reference standard to determine the parasite densities was not done because there were not enough samples for tests on gene deletions in these two study sites.

CHAPTER TWO

LITERATURE REVIEW

Malaria overview

The most clinically important of all protozoan diseases is malaria (Andrews, Fisher & Skinner-Adams, 2014). The disease is known to cause more harm to majority of infected people compared to all other infectious diseases (Gavazzi, Herrmann & Krause, 2004; Bernstein, 2014). In humans, five *Plasmodium* species are known to cause malaria. These are *P. falciparum*, *P. ovale*, *P. malariae*, *P. vivax* and the zoonotic form *P. knowlesi* (Rosenblatt, Reller & Weinstein, 2009).

Malaria parasites are transmitted to man from the bite of an infected female *Anopheles* mosquito during its blood meal. *Plasmodium* parasites are known to be transmitted nine to seventeen days after the female *Anopheles* mosquito picks up the *Plasmodium* parasite from the blood of an infected person and then transmits the parasite to naïve individual. Infections could be symptomatic or asymptomatic between eight and thirty days after mosquito bites depending on the *Plasmodium* species (Engleberg, DiRta & Dermody, 2007; Snow, Craig, Newton & Steketee, 2003).

The mosquito introduces the *Plasmodium* sporozoites during blood meal. After a time of pre-erythrocytic stage advancement within the hepatocytes, the

erythrocytic stage infection which causes the infection fever starts. The symptoms of the infection becomes apparent when the infection and fever reach the point where a minimum blood parasite density is attained. In non-susceptible individuals, the "pyrogenic density" is pegged at a normal of 10,000 p/μL with respect to *P. falciparum* and 200 p/μL for *P. vivax*, in spite of the fact that, people with much lower densities might be symptomatic (White, Dondorp & Paris, 2003).

Global Epidemiology of Malaria

Nearly 250 million individuals are affected by malaria resulting in nearly half a million deaths every year (WHO, 2017). Most tropical locales of the world are affected by malaria with *P. falciparum* prevailing in Haiti, Africa and Papua New Guinea while *P. vivax* is more typical of the Indian subcontinent and the Central Americas (WHO, 2016). The *P. malariae* is mostly endemic in all regions particularly all through sub-Saharan Africa. However, it is substantially less known than the other species. The *P. ovale* is moderately recorded outside Africa and it is less than 1% of the isolates in areas where it is found (White *et al.*, 2003).

More than a quarter of all malaria deaths that occur are found in Africa. One to three thousand children are killed by malaria every day in Africa. The one million deaths of malaria in Africa every year have 90% coming from sub-Saharan Africa are largely children under age five (WHO, 2016). In 2007, most pregnant women and their unborn children were vulnerable to malaria because of low birth weight and maternal anaemia. Children born to mothers who had

malaria were most likely to die in their first few months (UNICEF, 2007). However, this has improved in recent years (WHO, 2017). The global distribution of malaria has been presented in Figure 1.

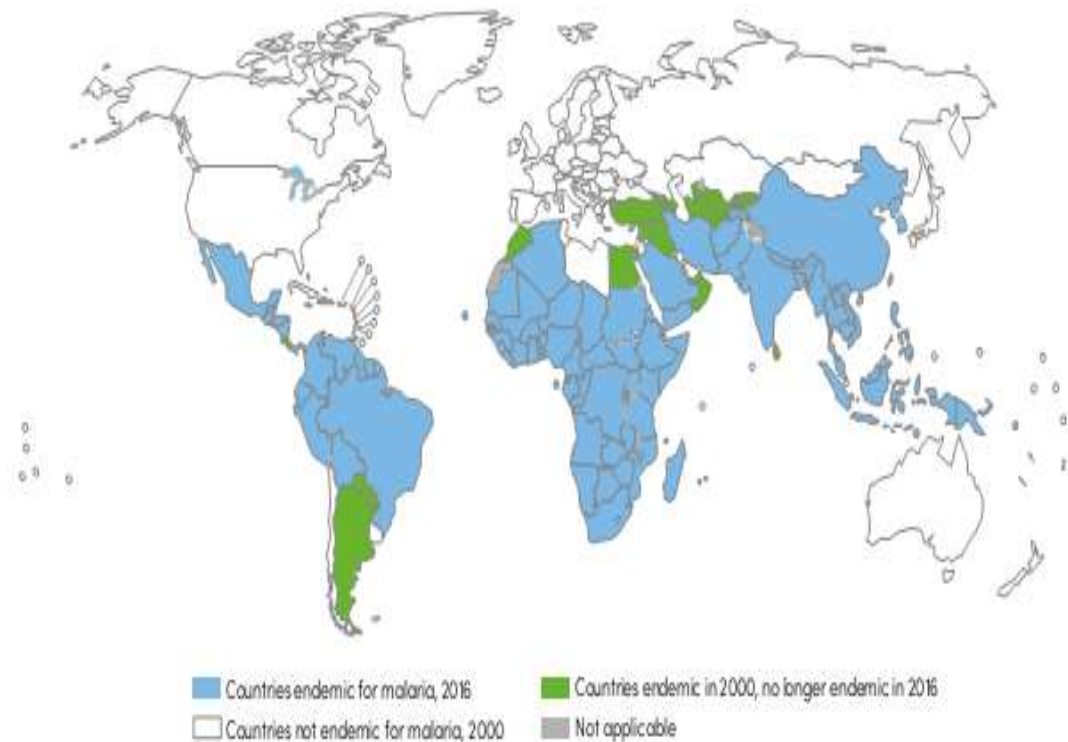


Figure 1: Global distribution of malaria, (WHO, 2017).

Source: [World malaria report, 2017](#).

Epidemiology of Malaria in Ghana

Malaria is a public health issue in Ghana so much that, in the first quarter of 2016, the Ghana Health Service (GHS) and Ministry of Health (MOH) through the District Health Information Management System (DHMIS) platform recorded 35.8 % of all Out Patient Department (OPD) cases were suspected to be malaria

in the first quarter of 2016 (DHMIS, 2016). In 2016, 79,822 out of the 356,209 OPD cases that were admitted into the wards were as a result of malaria, with majority of the malaria admission being children under age five (MOH, 2016). Figure 2 shows the parasite prevalence of *P. falciparum* infections in Ghana.

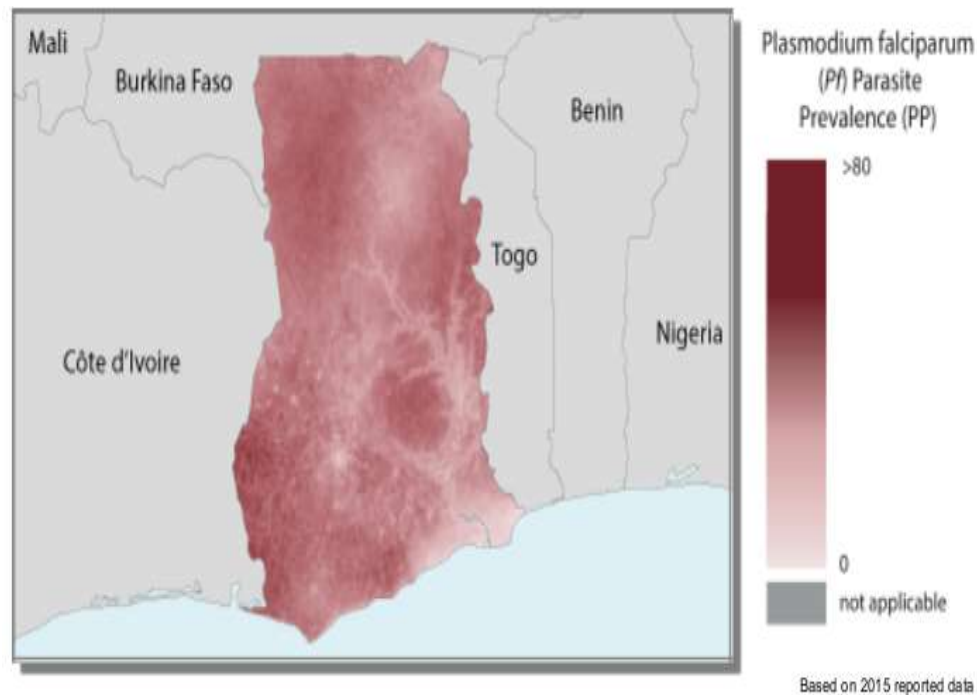


Figure 2: Map of *P. falciparum* Malaria prevalence in Ghana (WHO, 2016)

The total number of deaths that occurred during the first quarter of 2016 was 8,134 of which 4.7 % were caused by malaria (DHIMS, 2016). In the first quarter of 2017, the total number of OPD cases were 6,066,431 and 2,270,774 of these cases were suspected to be malaria. Out of the suspected malaria cases, 83.9 % of them were tested cases while 956,123 of these tested cases were actually positive for malaria by RDT and/or microscopy. The total number of deaths

recorded were 7,966 showing a 2.1 % reduction compared to the same period in the previous year (National Malaria Control Programme Bulletin, 2017).

Out of the first quarter hospital admissions of 2017, 69,467 cases were as a result of malaria. All deaths recorded in hospitals had 148 of them to be malaria and 74 of all malaria deaths were children under-five (Ghana Health Service, 2017). The case fatality rate of malaria in children under-five decreased from 0.39 to 0.22 during the first quarter of 2017. This showed a higher decrease of 105.4 % malaria deaths (DHIMS2, 2017).

Malaria testing rate in the first quarter of 2017 had increased compared to the same period in 2016 from 75.3 % to 83.9 %. However, microscopy positivity rate decreased from 21.7 % in 2016 to 21.4 % in 2017. RDT positivity increased from 20.6 % in the first quarter of 2016 to 21.5 % in 2017 (GHS, 2017).

Global Malaria Control

Various efforts to control malaria have been supported in the recent couple of years with expanded worldwide financing and more noteworthy political commitment. The burden of malaria is being reduced in various nations all through the world, amongst them are some nations in tropical Africa where the burden of the disease is most prominent (WHO, 2016).

These accomplishments have raised new hopes for the eradication of malaria. The WHO experts meeting pointed out the likelihood of attaining the objective of the Global Malaria Eradication Programme of the 1950s and the

1960s giving the recent insight on the effectiveness of intervention programmes as well as antimalarial tools which are being utilized worldwide (WHO, 2010).

The United States of America through the use of Dichlorodiphenyltrichloroethane (DDT) was able to eradicate female *Anopheles* mosquito and as a results the disease in the late 1940s and early 1950s. The world is still struggling to control the disease largely due to the ban on the usage of the DDT in recent times which has shown to be more poisonous. However, the world has witnessed a significant reduction in the huge morbidity and mortality that used to be associated with malaria (WHO, 2015).

Eradication of malaria requires a re-orientation of control measures, moving far from a populace based scope of interventions, to one in view of a program of compelling reconnaissance and reaction (Mendis, Rietveld, Warsame, Bosman & Greenwood, 2009).

More efforts will be required to prevent the resurgence of malaria from places where it has been eliminated. Eliminating malaria from nations where the intensity of transmission is high and stable, for example tropical Africa, will require more powerful tools such as vector eradication mechanisms and vaccine production and more grounded health frameworks than are accessible today (WHO, 2015). At the point when malaria is on the verge of eradication, the accomplishments should be solidified before a program re-introduction towards malaria disposal is considered. Malaria control and disposal are under the consistent danger of the parasite and vector mosquito creating resistance to drugs

and bug sprays, which are the foundations of current antimalarial medications. The prospects of malaria elimination, in this manner, lay vigorously on the results of research and advancement for better tools. Malaria control and eradication are complementary goals in the worldwide fight against malaria (Global Malaria Program, 2016; Mendis *et al.*, 2009).

Reports according to the GHS indicated that funding for malaria control in Ghana was provided by the Government, the Global fund, the World Bank, the United States President's Malaria Initiative and certain individual non-governmental organizations (NGOs) (GHS, 2013; 2014).

Life cycle of the Malaria Parasite *P. falciparum*

The life cycle of *P. falciparum* is known to consist of two hosts which are the human and the female *Anopheles* mosquito. The parasite revolves between these two organisms with the mosquito being a vector and human being host. This is because the mosquito is known to be only a carrier while man is known to be infected. The parasites reside in the female *Anopheles* mosquito's salivary gland as sporozoites (Ho, *et al*, 2014) and are released through a person's skin and into the bloodstream. It then migrates into the liver and the parasite then matures into schizonts within the liver's hepatocytes. In the liver and red blood cells (RBCs), they multiply asexually by binary fission. PfHRP2 is made by the parasite during the asexual stage and expressed on the surface of the infected RBCs (iRBCs) and is released into circulation during schizogony (Marquart, Butterworth, McCarthy

& Gatton, 2012). The schizonts get into the bloodstream as merozoites by which they can invade the RBCs.

Once in the RBCs, the parasites divide and mature within 2 days after which they cause the cells to burst releasing another generation of infective merozoites that can infect new RBCs (Engleberg *et al.*, 2007). Few merozoites then develop in the blood into male and female gametocytes, the sexual forms of the blood stage parasite. A mosquito picks male and female gametocytes during a blood meal for further transmission of the disease (White *et al.*, 2003).

Within the mosquito mid gut, male gametocytes lose their flagellum to become male gametes (haploid), which fertilize the female gametes (haploid) to produce the zygote (diploid). The zygote in the mid gut develops into an oocyst. Developed oocysts separate by meiosis to create sporozoite which migrates into the salivary glands and the cycle continues as shown in Figure 3 (Engleberg *et al.*, 2007).

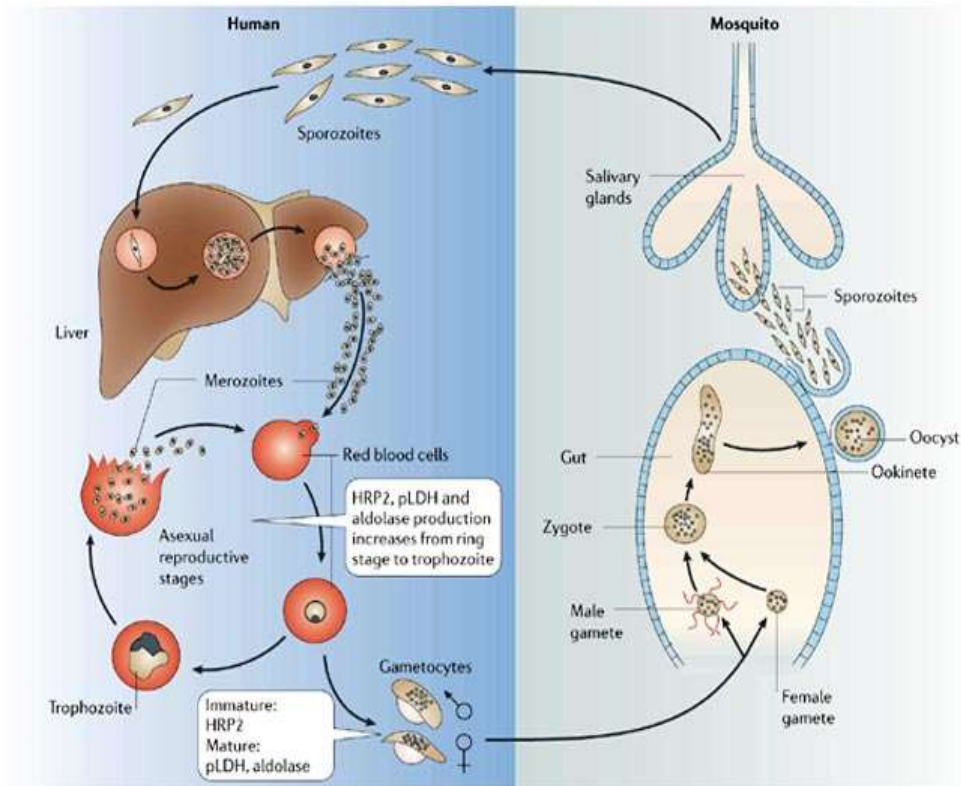


Figure 3: Life cycle of the malaria parasite *P. falciparum* showing the human phase and the vector female *Anopheles* mosquito phase. (Source: <https://i.pinimg.com/originals/f3/36/7e/f3367ec1f26cf6e34723402688b1ff8e.jpg>).

Clinical Symptoms and Infection Outcome

Pathological procedures in malaria relate completely to the intravascular erythrocytic disease (Winstanley *et al.*, 1992). The main side effects are not specific. There is frequently a prodrome stage where the patient feels unwell exhibiting symptoms of anorexia, fatigue, muscle throbbing and migraine. The fever and the compounding effects are non-specific so they resemble a flu-like sickness. In a few patients, stomach upset is noticeable and, albeit as a rule, the inside propensity is ordinary, at times looseness of the bowels might be an exhibiting highlight. The normal sharp increase of the fever and rigors, unmistakable in early portrayals of malaria, once in a while are observed today.

For the most part, the underlying fever, mostly, is sporadic. The clinical signs of malaria rely upon the level of background immunity (White *et al.*, 2003).

In zones of high transmission, clinical manifestations and signs are more likely to happen to children since mosquito bites are more likely to be a frequent occurrence. At lower transmission areas, a more extensive age range are easily susceptible, and insecure levels of transmission, or in non-immune travellers, malaria is suggestive and *P. falciparum* is largely fatal at all ages (Lacroix, Mukabana, Gouagna & Koella, 2005).

Pathogenesis of Malaria

Pathological processes in malaria relate entirely to the intravascular erythrocytic infection (White, 1999). The first symptoms are non-specific. These worsening symptoms and the accompanying fever are non-specific and resemble an influenza-like illness. In some patients abdominal pain is prominent. Although the bowel habit is usually normal, occasional diarrhoea may be a presenting feature (White *et al.*, 2003).

Severe Malaria

The *P. falciparum* is highly the most dangerous species amongst the *Plasmodia*, which can cause severe malaria. Severity of malaria is present with a single or more of the severe signs. These signs include severe anaemia which is less than 20 % Hb with parasite count greater than 100,000 parasites/ mm³), coma or severe prostration, severe jaundice (which is bilirubin greater than 2.5 mg/dl, acute renal failure which has greater than 3 mg/dl with urine less than 400 ml/day,

hypoglycaemia, shock of systolic pressure less than 80 mmHg with extremities, hyperparasitaemia, respiratory failure, hypoglycaemia (venous glucose less than 40 mg/dl), shock (systolic blood pressure less than 80 mmHg with extremities), (peripheral asexual stage parasitaemia greater than 10 %) or metabolic acidosis (peripheral venous lactate greater than 72 mg/dl, peripheral venous bicarbonate less than 270 mg/dl). (White *et al.*, 2003; CDC, 2016)

Uncomplicated Malaria

Uncomplicated malaria occurs barely affects people between 6-10 hours of infection and has been considered to have three stages. The cold stage with chills and shivering as the main symptoms, the hot stage with fever, headaches and vomiting as the main symptoms and the sweating stage which is mainly characterized by sweats, tiredness and return to normal temperatures.

This attack classically occurs on the second day of infection of the “tertian” parasites which are the *P. falciparum*, *P. vivax* and *P. ovale*) and the “quartan” parasite, *P. malariae* the third day of infection. During this stage, diagnosis can be done by microscopy with additional lab findings being mild anaemia, slightly decreased platelet counts and slightly high amounts of bilirubin. During this time, RDTs can also be used for its diagnosis (CDC, 2015).

Cerebral Malaria (CM)

Cerebral malaria according to the WHO has been given a strict definition for the sake of clarity and it is supposed to have the nearness of unarousable trance like state, prohibition of different encephalopathies and affirmation of the

P. falciparum infection. (WHO, 2007a). There should be *P. falciparum* parasitaemia of the patient and to be equal to Glasgow Coma Scale point 9 or less, with other causes such as hypoglycemia, bacterial meningitis and viral encephalitis ruled out.

To differentiate between CM and transient postictal coma, the patient should be unconscious for more than 30 minutes preceding a convulsion. A worse prognosis shows a deeper coma. In necessity, a lumbar puncture can be performed to rule out the possibility of a meningitis caused by a bacteria. However, all *P. falciparum* malaria patients with neurological symptoms of any sort have to be treated as CM cases (Trampuz *et al.* (2003).

Cerebral malaria is the type of malarial complication considered to cause death in severe *falciparum* infections. More than 80 % of all malaria deaths are because of the involvement of the central nervous system (CNS). Malaria fairly brings forth manifestations in the CNS and these could be attributed to the infection or its related high-grade fever and antimalarial drugs administered. Cerebral dysfunction may be manifested through any level of impaired consciousness, convulsions, delirium and overboard neurological characteristics.

In severe *falciparum* malaria infections, sudden manifestations of neurological disorders can happen following a seizure over a period of time. What causes neurological signs in malaria is mainly attributed to high level fever from the infection of *falciparum* malaria which can cause febrile convulsions in children and psychosis. Antimalarial drugs like quinine and mefloquine can cause

hallucinations and even altered behaviour during malaria treatments. Hypoglycaemia due to severe malaria or the effect of the drugs used to treat malaria can also present neurological manifestations. This is mostly common in pregnancy. Severe anaemia from an infection of *falciparum* malaria can cause cerebral dysfunction mostly in children. (WHO, 2007a).

Diagnosis

In the past, doctors used the physical symptoms of malaria to diagnose and prescribe treatments for patients presumed to have malaria. This was done without any tests performed. Eventually, confirmation of the presence of parasites by Giemsa stained microscopic slides was added onto the presumptive diagnosis. However, microscopy was only performed where there was an expert microscopist and resources available. Health experts still diagnose malaria basically using clinical assessment of side effects where microscopy is unavailable (Baker *et al*, 2010).

Presently, RDTs for malaria diagnosis has taken over thick and thin film blood smears (light microscopy). In villages and semi urban areas, which lack microscopes, trained work force and experience frequent power outages, RDTs have become essential diagnostic tools.

Varying patterns of internationally accepted morphological appearances of *Plasmodium* species, conceivably because of medication pressure, strain variety, or ways to deal with blood collection, have made diagnostic issues very difficult to be settled just by reference to an atlas of parasitology (Moody, 2002). This,

however, has led to difficult diagnosis in malaria. As a result of the restrictions of microscopy for malaria determination, worldwide malaria control programs have concentrated on the improvement of other diagnostic tests that can be utilized as a part of field diagnosis (Wilson, 2012).

Microscopy

This is known as the “Gold Standard” for the identification of species specificity and all the different stages for the *Plasmodium* parasite. It is by far the most widely used method of diagnosis for malaria (Azikiwe, Ifezulike, Siminialayi, Amazu, Enye & Nwakwunite, 2012; Falade *et al.*, 2016). In 2014, the WHO report showed the use of microscopy for diagnosis hit a maximum high of 203 million cases (WHO, 2016).

It is a recommendation of WHO that at least microscopy or RDT should be used in the diagnosis of malaria (WHO, 2015). However, there are some limitations to how accurate a microscopy diagnosis could be in primary health care levels. Some of these limitations can range from the low limit of detection (~ 50 parasite/ microliter) deficiencies in personnel (inadequate training of personnel) to substandard equipment and inadequate flow of reagents and materials with good shelf life (Payne, 1988).

To address the impediments of microscopy, PCR-based procedures are used and different strategies are being investigated. To be approved, these techniques must be benchmarked against microscopy or PCR investigation and against reference strains (Mouatcho & Dean Goldring, 2013).

Serology

Serology distinguishes antibodies against malarial parasite antigens, utilizing either indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA). Serology allows for the pickup current infection and also measures past exposures to the disease. This normally detects the exposure to the organism but not its presence (Rosenblatt *et al.*, 2009).

Babesia bovis antigen studies showed positive RDTs even though it was negative from PCR and other tests. These samples were later found to have recent malaria past infection. The IgM and IgG subclasses of the malaria infection were still in the blood sample used (Rios, Alvarez & Blair, 2003). This brought to light the cross reactivity of Babesia and *P. falciparum*. In other Babesia studies, malaria was found to be positive in IFA test but was negative in ELISA. The sample used were samples which have been confirmed to have current infection with very low parasitaemia. This reiterates that IFA may not be reliable for current infection diagnosis (James *et al.*, 1987).

Gel Diffusion Tests

Gel diffusion tests entails diffusing malaria antigens which are soluble into an agar gel against a serum containing antibodies. Immunoprecipitation occurs when an antigen chances upon its specific precipitation antibody and forms a precipitin line. Mostly, antigens from the asexual stage of the *P. falciparum* are used.

The use of gel diffusion test is advantageous because it does not require complex equipment in its use. Also very large numbers of antiseras can be

diagnosed in a day for the presence or absence of precipitins. It can also be used to detect the presence of soluble malaria antigens in different sera. This method is best used in high endemic areas because the test may give poor results in lower transmission areas where antibody concentrations in the serum are generally very low (Balows, William Jr., Ohashi & Turano, 2012; Claessens, Affara, Assefa, Kwiatkowski & Conway, 2017; Druilhe & Monjour, 1975; Srivastava *et al.*, 1989).

Indirect Haemagglutination Tests (IHA)

Indirect haemagglutination test may be likened to an ELISA, however, agglutination of cells are expected in the wells instead of colorations. Control antigens and antigens prepared are added to sensitized cells which have been fixated (fixated RBCs). These fixated cells are then treated with tannic acid and antigenitrations are done. Samples are examined by doing various dilutions with intermittent shaking. Results are read looking for agglutination in wells of positive control and compared with that of the samples (Bulletin of WHO, 1974). Indirect haemagglutination test is highly sensitive and scarcely produce false positives and is easily done without any complex equipment. Early infection, however, may produce false negatives though the parasite may be present (Ambroise-Thomas, 1978; Sulzer, Glosser, Rogers, Jones & Frix, 1975; Mathews *et al.*, 1975).

Immunofluorescence Assay (IFA)

The immunofluorescent antibody test is an appropriate and reproducible method for looking at antibody levels for malaria infections, both past and present

infections (Sulzer, Wilson & Hall, 1969). Immunofluorescence antibody assays have been used around the world for malaria diagnosis.

Duo-Quan and the colleagues in 2009 in the China Yangtze Three Gorges Project (TGP) used IFA to ascertain the presence of malaria in the Gorge Dam area, however, there was no history of malaria in the area prior to the project. For instance, in France, IFA is combined with donor questionnaire as part of a targeted screening strategy (Tangpukdee, Duangdee, Wilairatana & Krudsood, 2009; Duo-Quan, Lin-Hua, Zhen-Cheng, Xiang & Man-Ni, 2009). In Stockholm University, Department of Immunology, IFA was done on glutaraldehyde-fixed monolayers of F32 *P. falciparum*-infected erythrocytes. (Iqbal, Sher & Rab, 2000).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA has been used to quantify parasite biomarkers such as *Plasmodium* Topoisomerase I and Tyrosyl-DNA Phosphodiesterase I in whole blood (Hede *et al.*, 2018). A parasite biomarker of choice is used to estimate the level of infection that can be found in whole blood or serum or plasma. This can be done irrespective of the parasites' location in the body. The best biomarkers used are mostly the markers used for RDTs (Moody, 2002). Evidence available shows that PfHRP2 based assays are the most sensitive in the *P. falciparum* detection. Some studies have also showed that *pfhrp2* have been used in detecting the presence of the parasite in placental malaria cases (Leke *et al.*, 1999). ELISA is mostly used to detect very low levels of infection that is undetectable in RDTs and because of

the high throughput nature, it can be very useful in epidemiological studies (Kifude *et al.*, 2008).

Rapid Diagnostic Tests (RDTs) for Malaria

The RDT for malaria, also known as “dipstick/malaria rapid diagnostic device” detects particular antigenic proteins that *Plasmodia* produce. These proteins can be found in the body fluids (mostly blood or saliva) of infected individuals. The RDTs function by the lateral flow method or the Immunochromatographic Strip (ICS) method which shows availability of the antigenic proteins by changing colour on the absorbent nitrocellulose strip (Figure 4). There are RDTs which detect antibodies of antimalarial proteins, however, they are mostly used in case management. For instance, during blood donation, it can be used to screen donated blood to prevent introduction of malaria into transfused patients (Moody, 2002). RDTs are mostly useful in rural healthcare areas where there is lack of trained personnel for microscopy as well as unavailability of microscopes. Diagnosis becomes easy in these areas.

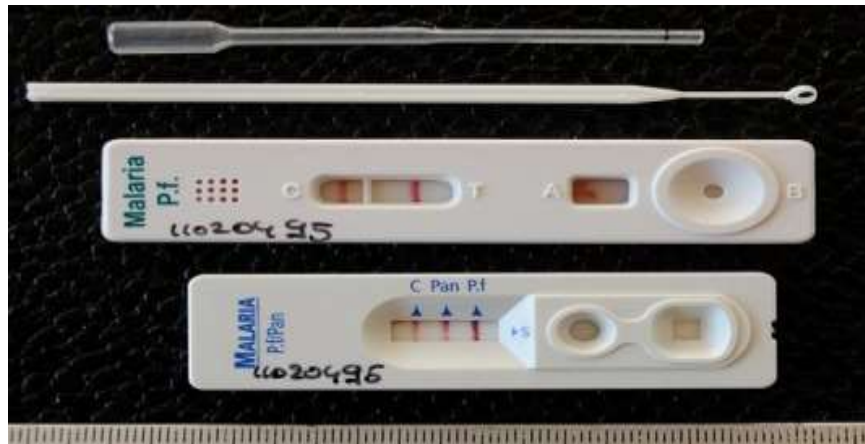


Figure 4: PfHRP2 and PfHRP2/Pan specific RDTs positive for *P. falciparum* or mixed infection Source: <http://www.malwest.gr/en-us/malaria/informationforhealthcareprofessionals/laboratorydiagnosis.aspx>

Mechanism of action of Rapid diagnostic tests

1. Dye-labelled antibody, specific for target antigen, is present on the lower end of nitrocellulose strip or in a plastic well provided with the strip. A specific antibody for the target antigen is bound to the strip in a thin (test) line. Either an antibody specific for a labelled antigen or an antigen for a labelled antibody is bound at the control line (Figure 5).
2. Blood and buffer, which have been placed on strip or in the well, are mixed with labelled antibody and are drawn up strip across the lines of bound antibody
3. If antigen is present, some labelled antibody will be trapped on the test line. The excess labelled-antibody binds to the control line.

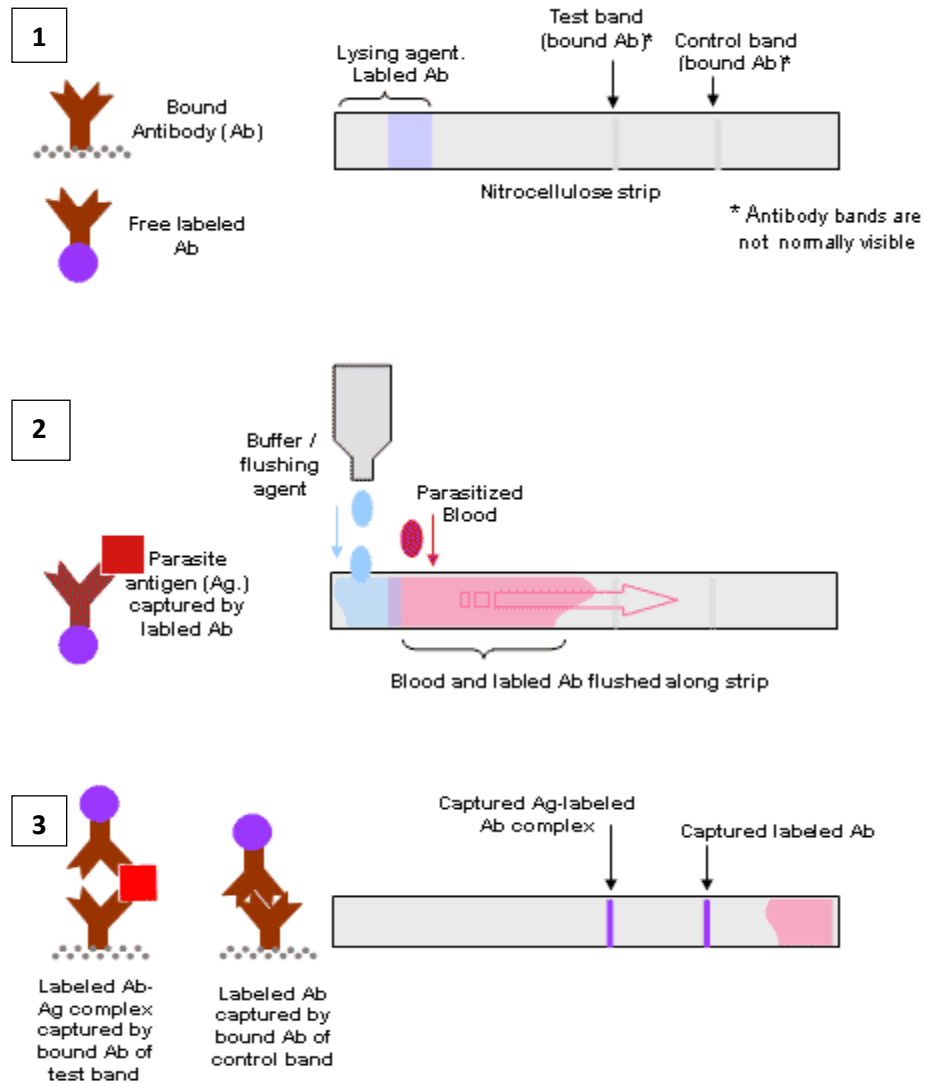


Figure 5: Malaria Rapid Diagnostic Test Performance Results of WHO product testing of malaria RDTs: Round 3 (2010-2011) Source: WHO/FIND/CDC/TDR (2011)

Types of malaria RDTs

Rapid diagnostic tests kits are mostly categorized into two. The dipstick (test strips) put in tubes having unclotted blood and/or buffer and the nitrocellulose strips which are mostly put in cards or cassettes. Even though cards

and cassettes are expensive, they are quite easier to use. These two types basically work on the principle of capillary flow motions.

The Dipstick RDT strips

The dipstick format have been reported to have binding sites to test for multiple antigens by placing the dipstick in the sample within a tube Jelinek *et al.*, 1999). Jelinek and other colleagues also reported dipsticks to have the potential of enhancing *P. falciparum* diagnosis in nonspecialized labs. They also reiterated that, travelers should only use dipstick if they had been trained and appropriately instructed on the performance of the test procedure (Jelinek, Grobusch & Nothdurft, 2000). They are not common in these days since their specificity and sensitivity was not as the lateral flow type (Maltha, Gillet & Jacobs, 2013). This made the nitrocellulose membrane strips more prominent and produced by many companies recently.

The nitrocellulose membrane RDT strips

The nitrocellulose membrane RDT strips is the most widely used strip with over 60 companies producing them (Wongschangarai *et al.*, 2007). On one end of the nitrocellulose strip, one or two indicator-labeled antibodies, one specific for each target antigen, are placed. A second antibody specific for a different epitope of each of the target antigens is bound to the strip in a thin line. Another antibody specific for the indicator-labeled antibody complex is bound to the control line. Blood and buffer are added to the strip where the lysing agent and labeled antibody are located and are drawn up the strip. If antigen is present, some

indicator-labeled antibody-antigen complexes will be trapped on the test line and become visible. Additional indicator-labeled antibody is trapped on the control line and becomes visible as shown in Figure 6.

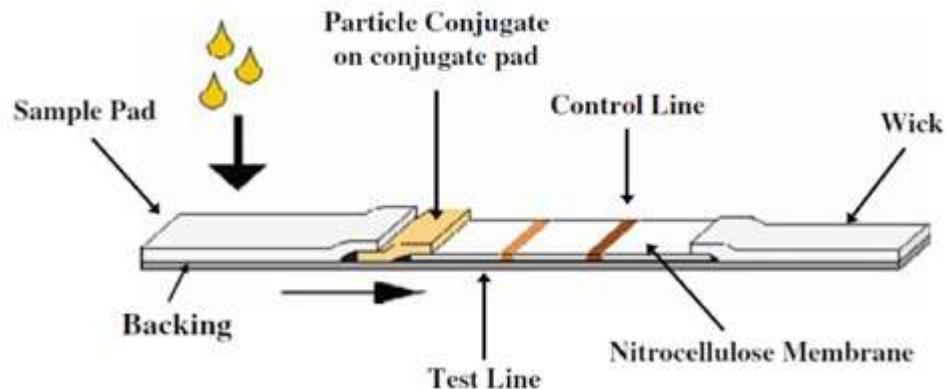


Figure 6: Schematic diagram of a nitrocellulose Membrane RDT. Source: <https://www.creative-diagnostics.com/Immunochromatography-guide.htm>

Basically, RDTs that are commercially made detect three main types of antigens. These are the *Plasmodium* histidine-rich protein II (PfHRP2), *Plasmodium* Lactate Dehydrogenase (pLDH), *Plasmodium* Aldolase (pAldo) which is pan-specific and the *Plasmodium* glutamate dehydrogenase (pGluDH) which is not widely used.

***Plasmodium* histidine-rich protein II (PfHRP2)**

Histidine-rich protein II is specific to *P. falciparum* and is the one known to be heat stable antigen and highly abundant soluble protein found within membrane and cytoplasm of iRBCs. This type of protein is the most used because of its qualities. It is known to be still active over long travels (WHO, 2004; Hänscheid, 2003). The antigen is expressed on the surface of the RBC membranes

and are produced by the parasite's asexual stage and gametocytes. They are the most persistent antigen known to remain in the blood for a minimum of 28 days (Beshir *et al.*, 2017; Dorado *et al.*, 2016; Hendriksen *et al.*, 2011). This brings about false positives even after parasite clearance. There are some parasite strains which lack the *pfhrp2* gene and thus do not produce this antigen of interest. This produces false negatives. The mere presence of the *pfhrp2* deletants affect RDT accuracy in endemic regions and these would pose significant implications on RDT implementation, clinical case managements and efforts to control malaria (Gatton *et al.*, 2017).

***Plasmodium* Lactate Dehydrogenase (pLDH)**

Plasmodium Lactate Dehydrogenase (pLDH) is produced by the trophozoite stage and gametocyte stage of all the *Plasmodia*. It is only present and released in iRBCs. The most readily available pLDH RDTs are the *P. falciparum* specific and the pan-specific for all the common human infective *Plasmodia*. Some pLDH RDTs are also *P. vivax* specific (Manjula, Soumya & Majigoudar, 2014). Since pLDH is produced by living parasites, the test is used to differentiate between treated and untreated malaria infections. Due to this, they are mainly used for follow up malaria cases. These RDTs are more expensive and less stable at high humidity and temperature (Simpalipan, Pattaradilokrat & Harnyuttanakorn, 2018). These have greatly been used in recent times but have issues in the *P. vivax* specific types. According to a study, pLDH and pAldo antigens RDTs worked differently in *P. vivax* samples. This could bring about misdiagnosis (Dzakah *et*

al., 2014). Combination of the antigens into one RDT for diagnosis is preferred for all the species of *Plasmodium*.

***Plasmodium* Aldolase (pAldo is pan-specific)**

Plasmodium Aldolase are antigens which are major enzymatic proteins conserved in malaria parasite's glycolytic pathway and are soluble as well as abundantly found within the parasite (WHO, 2006). These pAldo antigens are mostly seen produced together with the HRP2 on the same test kit. BinaxNOW® in the US mostly produce the most efficient malarial pan-specific RDT kits (Rosenblatt *et al.*, 2009). Though this is pan-specific, studies have shown it to be more accurate in sensitivity and specificity than the PfHRP2 but also has an issue with detecting limit (Dzakah *et al.*, 2013).

***Plasmodium* glutamate dehydrogenase (pGluDH)**

The pGluDH is an enzyme given as a marker enzyme for *Plasmodium* species which cannot be found in the host RBC (Picard Maureau *et al.*, 1975).

It was discovered to be very appropriate for routine and standard malarial testing. The existence of pGluDH has been reported to be parasite viable and are therefore suitable for rapid diagnostic testing. The antigen is said to have the ability to distinguish between live and dead malaria parasites. A pGluDH RDT was developed in China in the early 2000s (Li *et al.*, 2005).

GluDHs are ubiquitous enzymes that occupy an important branch-point between carbon and nitrogen metabolism. Both nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) dependent

GluDH enzymes are present in all the *Plasmodium* species, however, the NAD-dependent GluDH is relatively unstable and not useful for diagnostic purposes. Glutamate dehydrogenase provides an oxidizable carbon source used for the production of energy as well as a reduced electron carrier, NADH. Glutamate is a principal amino donor to other amino acids in subsequent transamination reactions. The multiple roles of glutamate in nitrogen balance make it a gateway between free ammonia and the amino groups of most amino acids (Jain *et al.*, 2014).

Though pGluDH has been known to be a good RDT candidate, its RDTs for malaria detection have not been easily accessible (Ishengoma *et al.*, 2016).

Importance of RDTs on malaria diagnosis

Within the last 10 years, malaria diagnosis has been evolving rapidly mostly because of the introduction of RDTs in malaria diagnosis (McMorrow, Aidoo & Kachur, 2011). Very accurate diagnosis is fast becoming an all-important venture in disease surveillance and case managements. The WHO recommended all malaria suspected cases to be confirmed by the use of a diagnostic assay of the parasites (WHO, 2010). It was a sharp contrast to the earlier policy that recommended the universal treatment of children under-five in endemic areas and empirical treatment for adults in non-laboratory testing areas (WHO, 2006). Expansion capacity of malaria diagnosis over the years have shown that there are possibilities of reducing the inappropriate use of antimalarial drugs (McMorrow *et al.*, 2011).

Rapid diagnostic tests have many advantages over microscopy and clinical diagnosis. Rapid diagnostic tests in field trials, have shown to have greater than 90 % specificity and sensitivity for *P. falciparum* infection with ≥ 200 parasites/ μL (Hopkins, Kambale, Kanya, Staedke, Dorsey & Rosenthal, 2007). Among all the malaria diagnostic tests, RDTs are the simplest. This is because they do not need the use of any sophisticated equipment nor electricity. Their results are made ready in less than 20 minutes. They are relatively cheaper with price range of \$0.60 to \$1.2 per a single test (Moody, 2002). Since RDTs are the most simple to use, clinicians, health workers and volunteers in the community can be taught to perform them within a few hours of practical training. Correct interpretation of RDTs results is less subjective than that of microscopy which requires a lot of skill. On a RDT, there is a line present or absent on the test kit (Proux *et al.*, 2001).

The RDTs on the market now can recognize one or more of the target antigens. HRP2-based tests are highly specific and sensitive for *P. falciparum*. The pLDH-detected tests are specific for *P. falciparum* or *P. vivax* and/or pan-specific for the detection of all malaria species. Very few commercially available RDTs use *Plasmodium* aldolase as a pan-species target antigen. Malaria RDTs have shelf-life of 18 – 24 months, which allows enough time for delivery, distribution, and use in almost all settings (Moody, 2002).

Combining accurate and early diagnosis using RDTs, fast treatments with the ACTs by Community Health workers have shown great importance in reducing the incidence and death rate of malaria in low transmission areas

(Hopkins *et al.*, 2007). However, there are few limitations of the RDTs. These include the level of capture (greater than or equal to 200 parasites per microliter), storage in temperate conditions, parasite densities determination and deletions of the *pfhrp2/pfhrp3* gene segments which cause negative results in some cases even when the parasite is present (WHO, 2016).

Molecular Diagnostics

Molecular techniques in diagnostics have been known to have higher sensitivities than the RDTs and the best microscopist. Averagely, microscopists, detect 50 parasites per microliter (p/μL) of blood while an expert may identify ≥ 20 regular infections (Chiodini, 2014). The detection limit of RDTs are mostly 100p/μL while PCRs have detection limits of < 5 p/μL (Erdman & Kain, 2008) and RT-PCR less than 1 p/μL. A number of different molecular diagnostic tools can be used for malaria diagnosis, such as PCR, which have many different types, the loop-mediated isothermal amplification and the quantitative or realtime nucleic acid-based amplification. .

Polymerase Chain Reaction

Even though microscopy is the gold standard for malaria diagnosis and species identification in clinical settings (Makler, Palmer & Ager, 1998; Warhurst & Williams, 1996), PCR has been reported to have 100 times greater sensitivity and specificity (Milne, Kyi, Chiodini & Warhurst, 1994; Snounou *et al.*, 1993). Compared with microscopy, PCR is more sensitive and can be used in low transmission settings and to detect subclinical infections. However, reagent and equipment costs as well as quality assurance have made PCR-based diagnostics

limited to research labs, (Coleman *et al.*, 2006; Roshanravan *et al.*, 2003). Gal and the colleagues reported that serum and plasma give better DNA yield for PCR than whole blood (Gal *et al.*, 2001). This was confirmed in another study on stored serum samples (Bharti *et al.*, 2007). This allowed testing stored sera samples to determine malaria infection rates in a sensitive manner. Though whole blood was used, test sensitivity is still seen to be high (Amoah *et al.*, 2016; Kattenberg *et al.*, 2012; Snounou *et al.*, 1999).

Nested PCR

Nested PCR is a modified PCR designed to improve on the sensitivity and specificity of reaction outcomes. This requires two primer sets in two consecutive PCR reactions. The first set of primers used in an initial PCR reaction are designed to anneal to sequences upstream from the second set of primers. Amplicons from the first PCR reaction are used as the template DNA for the second set of primers and its second PCR amplification step (Haddad *et al.*, 1999).

Sensitivity and specificity of DNA amplification are significantly enhanced with this technique. Nonetheless, there is a risk of contamination from carryover amplicon products. To be able to reduce carryover contaminations, the two processes can be done in two separate rooms or areas. Amplicons from nested PCR assays are viewed in the same manner as in conventional PCR (Buttler, 2012; Carr, William & Hayden, 2010).

In areas where other species apart from *P. falciparum* are present, nested PCR is very useful for malarial diagnosis to complement microscopy (Zakeri, Najafabadi, Zare & Djadid, 2002).

Real time Quantitative PCR

Quantitative polymerase chain reaction (qPCR) is a method by which the amount of the PCR product can be determined, in real-time. It is very useful for investigating gene expression. The process is often referred to as real-time PCR or real-time quantitative PCR (qRT-PCR) (Heid, Stevens, Livak & Williams, 1996), or depending on the application, quantitative reverse-transcriptase PCR (both of which are abbreviated to RT-PCR) (Lobert, Hiser & Correia, 2010).

Quantitative PCR does not rely on any downstream analysis such as electrophoresis or densitometry. It is very versatile, allowing multiple PCR targets to be assessed concurrently. This can be a little trickier to set up than “normal” PCR. However, familiarity with “normal” PCR makes one able to successfully undertake Q-PCR (Maddocks & Jenkins, 2016); Brazeau & Brazeau, 2013).

Some specimens were identified by sequencing pan-plasmodium *18s rRNA* PCR products and two of these samples were seen to have mixed infection after a real-time PCR assay was done. This assay could therefore be integrated into malaria testing algorithm in low transmission areas. This will give way for definitive species identification after microscopy (Lefterova, Budvytiene, Sandlund, Färnert & Banaei, 2015). This is an easy parasite identification procedure but highly expensive with sophisticated equipment.

Multiplex PCR

Multiplex PCR (M-PCR) is a variation of the conventional PCR. The basic principle of multiplex PCR is the same as that of the conventional PCR, except that more than one pair of primers are required in the same reaction. The primers can specifically combine with their corresponding DNA template, and more than one DNA fragment will be amplified in one reaction simultaneously. The faster and more economic multiplex PCR has similar specificity and sensitivity as simplex PCR and shows greater flexibility in the design of primers and PCR reaction conditions (Shang, Xu, Wang, Xu & Huang, 2017). Multiplex PCR could provide internal controls and appreciation of the quantity and quality of amplified templates (Xu, Huang, Wang, Zhang & Luo, 2006).

Though multiplex PCR has a lot of merits, its limitations cannot be overlooked. The self-inhibition among different sets of primers, low amplification efficiency and no identical efficiency on different templates are worrying. These reasons would restrict its further development and broad application (Nikiforova, LaFramboise & Nikiforov, 2015).

In comparing realtime PCR to PlasmoNex Multiplex PCR Kit and *MSP*-multiplex PCR, the *MSP*-multiplex PCR assay performed slightly better than realtime multiplex PCR and the PlasmoNex Multiplex PCR Kit with regard to *P. ovale* in terms of detection limit. *P. falciparum* detection in both multiplex were however, poorly sensitive as compared to real-time PCR (Lau *et al.*, 2015). This showed that multiplex could not be used in large diagnostic settings where *P. falciparum* is endemic.

Comparing qPCR to microscopy, Reller *et al* (2013) used a high-throughput multiplex 5' nuclease qPCR assays to distinguish between the five human infective *Plasmodium* species. This yielded a good result since the assay was able to identify all the confirmed microscopy positives. This showed that multiplex PCR is a good method for malarial species identification in large studies.

Loop-mediated isothermal amplification (LAMP)

One main set back of PCR for malaria diagnosis, despite its high sensitivity is that it requires very sophisticated equipment, laboratory infrastructure and training and also a long time to obtain results. This makes the techniques more expensive and challenging to do in field work and clinical labs, (Lucchi *et al.*, 2010). To be able to better control malaria and eventually eradicate it, very sensitive inexpensive diagnostic tools are required to detect very low levels of parasitaemia in asymptomatic persons. For this reason, other molecular techniques for field use needed to be developed to complement or replace the existing methods for malaria diagnosis and control.

Loop-mediated isothermal amplification is a relatively simpler and field adaptable alternative to PCR (Notomi *et al.*, 2000). In this technique, parasite DNA is amplified with strand displacement properties under isothermal conditions, therefore, no sophisticated equipment and thermal cyclers are required. Amplified DNA forms magnesium pyrophosphate which appears as a

precipitate during the progression of the reaction and serves as an indication of parasite positivity.

Loop-mediated isothermal amplification exhibits high efficiency in DNA amplification, amplifying 10^9 copies in less than an hour. Four different primers are required for LAMP, however, in malaria diagnosis six are used. These primers are specific to six sites of *P. falciparum*, making them highly specific. The addition of two loop-primers accelerates the time for product formation (Nagamine, Hase & Notomi, 2002), which shortens the reaction time. Since this method does not need a thermocycler or any sophisticated equipment and training, it could be used as a point-of-care (POC) molecular diagnosis in field works. LAMP has been used in the diagnosis of other infectious diseases like the *Legionella* bacteria, West Nile Virus, severe acute respiratory syndrome, avian influenza virus and norovirus (Annaka, 2003; Parida, Posadas, Inoue, Hasebe & Morita, 2004; Imai *et al.*, 2006; Fukuda, Takao, Kuwayama, Shimazu & Miyazaki, 2006).

Within the last fifteen years, LAMP method have been used for the detection of *Plasmodium* infection with the *18s rRNA* gene as target gene (Zheng, Xie & Chen, 2002; Han *et al.*, 2007; Paris *et al.*, 2007; Yamamura, Makimura & Ota, 2009). The first time LAMP was reportedly successful in the diagnosis of malaria was in 2006 (Poon *et al.*, 2006). They detected *P. falciparum* from heat-treated clinical samples which had their packed red cells boiled at 99°C for 10

minutes. The cells were then pelleted by centrifugation the supernatant were then used in the LAMP assay.

Lucchi *et al* (2010) reported LAMP sensitivity of 95 % and specificity of 99 % compared to nested PCR. Han *et al* (2007) used conventional DNA extraction method before a species specific LAMP diagnosis and reported 98.5 % sensitivity against 94.3 % specificity compared to microscopy and nested PCR. The limits of detection of the target *18s rRNA* genes for *P. ovale* and *P. malariae* were 10 copies while that of *P. falciparum* and *P. vivax* were 100 copies (Han *et al*).

In others studies, when LAMP was compared with both microscopy and HRP2 RDT, LAMP had 100 % specificity relative to both (HRP2 RDT and microscopy) and sensitivity of 73.1 % and 77.6% relative to microscopy and RDT respectively. Nested PCR-based on primers designed by Singh *et al* (1999) showed that LAMP had sensitivity of 79.1% against 58.3% specificity (Poon *et al.*, 2006; Paris *et al.*, 2007). This was after heat treatment for DNA extraction was used (Singh *et al.*, 1999). Zheng and others used *P. vivax* primers to detect microscopy positive clinical samples using LAMP assay. The detection limit of *P. vivax* showed 30 parasites per microliter (30p/μL) with 100% specificity and 98.3% sensitivity compared to microscopy (Zheng, Xie & Chen, 2002). This shows that there are other equally important and good methods for malaria diagnosis, each with their own set of limitations. The LAMP can be used in field studies to confirm PfHRP2 RDT negatives and positives.

Nucleic Acid Sequence-based Amplification (NASBA)

Nucleic acid sequence-based amplification is a new technique which uses isothermal processes and RNA polymerase to amplify RNA. It has been reported as sensitive, transcription-based amplification system specifically designed for the identification of RNA targets. However in other NASBAs, some DNA may be amplified but is highly inefficient when compared to that of the RNA (Fakkrudin, Mazumdar, Chowdhury & Mannan, 2012).

Nucleic Acid sequence-based amplification is primer-dependent and amplicon detection is based on probe binding, primer and probe design rules are included. For the isolation of nucleic acids prior to NASBA, the method, based on the denaturing properties of guanidine isothiocyanate and binding of nucleic acid to silica particles, is preferred. At the moment, electro-chemiluminescence (ECL) is used to detect the amplicon at the end of amplification. In an HIV-1 quantitative NASBA, 48 samples were detected in 90 minutes (Deiman, van Aarle & Sillekens, 2002; Yoo *et al.*, 2005).

Schallig *et al* (2003) used NASBA to diagnose malaria and reported that NASBA could be helpful in areas of low parasitaemia. Comparison of the parasite densities obtained by microscopy and QT-NASBA with 120 blood samples from Kenyan patients with clinical malaria revealed that for 93% of the results were within a 1-log difference. QT-NASBA is there a very useful tool for the detection of low parasite levels in patients with early-stage malaria and for the monitoring of the efficacy of drug treatment (Schoone, Oskam, Kroon, Schallig & Omar, 2000).

Plasmodium Genes and Antigens for malaria diagnoses

Plasmodium falciparum *hrp2* gene

Histidine-rich protein II gene (*pfhrp2*) is a gene located sub telomerically on the eighth chromosome of the malaria parasite (Iqbal *et al.*, 2004). The PfHRP2 is a *P. falciparum*-specific water-soluble protein, localized in the parasite cytoplasm and on the surface membrane of infected erythrocyte. It is found on protruded knobs which is known to account for the sequestration of the trophozoites and schizonts in post capillary venules (Pain *et al.*, 2014).

The genes have hydrophobic signal peptides (brown), an intervening intron, and an extensive region of tandem repeats (pink). The high homology (85–90%) between the tandem repeat domains and the flanking regions of the repeats of *hrp* II and *hrp* III genes is shown in Figure 7 (Sullivan, Ayala & Goldberg, 1996; Jain *et al.*, 2014). This explains why the *pfhrp3* gene substitutes for the *pfhrp2* gene when it has been deleted. Their high homology gives room for overlapping and replacement functions.

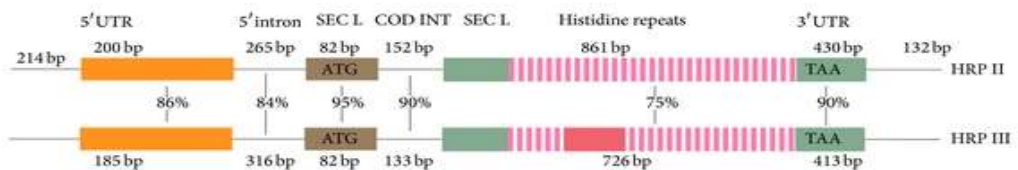


Figure 7: Schematic alignment of *hrp* II and *hrp* III genes including 5' and 3' untranslated region (UTRs), (Source: Pain *et al.*, 2014).

NB* INT = intron, SEC = secretory leader.

The various ways by which *pfhrp2* deletions are possible have been presented in Figure 8. The entire gene of *pfhrp2* can be deleted while part of it may be deleted as well. There can be deletion of exon 1, deletion of exon 2, deletion of the flanking genes and deletion of any two of these genes as well as deletion of the entire *pfhrp2* gene.

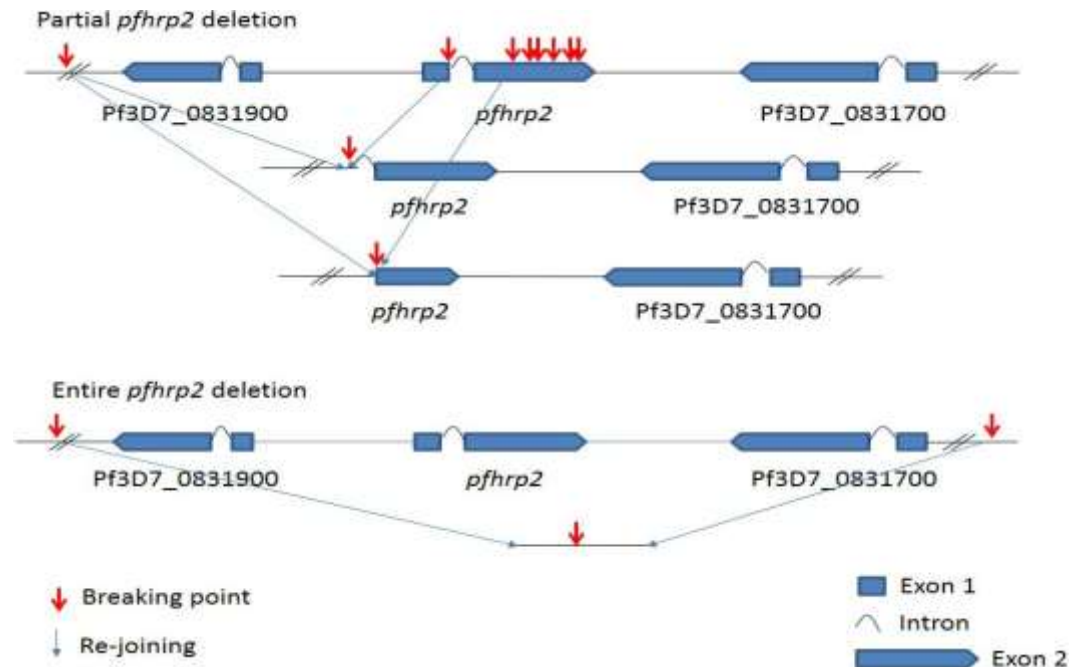


Figure 8: Schematic presentation of various breaking and rejoining points of the *pfhrp2* gene to show how *pfhrp2* is deleted (Source: Chenq *et al.*, 2014).

The PfHRP2 antigen concentrations increase as the parasite grows from the ring stage to the trophozoite stage and it easily diffuses into the plasma. (Howard *et al.*, 1986; Rock *et al.*, 1987). These PfHRP2 antigens are mostly found in the asexual stages, but are also found in young gametocytes of *P. falciparum*. This could be the reason why detection is possible at lower parasite densities and also its detectability even after 28 days of clinical presentations and total clearance of the parasite from the patients system (Murray & Bennett, 2009). The *pfhrp2* mutants can escape recognition by monoclonal antibodies and may be

responsible for false negative tests (Baker *et al.*, 2005; Lee *et al.*, 2006). Findings about PfHRP2 from 19 different countries have revealed that only 84% of *P. falciparum* have been detected (Murray & Bennett, 2009).

Many reports have shown that the main function of *hrp2* is heme binding and that its role is linked to the heme detoxification in malaria parasites. *Pfhrp2* is also known to initiate hemozoin formation. PfHRP2 has been suggested to play an important role of a buffering protein since it helps the parasite to stabilize the changes to the cytoskeleton induced by other released parasitic proteins. (Jain, Chakma, Patra & Goswami, 2014)

***Plasmodium falciparum* hrp3 gene**

Histidine-rich protein III (*pfhrp3*) also known as small histidine-alanine-rich protein (SHARP) is a smaller protein and has been reported to be as abundant as the *hrp2* protein. The *hrp3* gene is located subtelomerically on the thirteenth chromosome of the *P. falciparum* malaria parasite genome. The known *pfhrp3* sequence was taken from FC27 strain of the *P. falciparum* isolates from Papua New Guinea (Stahl *et al.*, 1985).

The *pfhrp3* gene has however been reported to be similar in structural homology with the *pfhrp2* and as a result, it cross reacts with *pfhrp2* antibody coated RDTs. These *pfhrp3* genes have therefore been seen to contribute to the diagnosis of *P. falciparum* malaria (Kumar *et al.*, 2012). Many studies have been done to show that there are similarities in both function and structure of both *pfhrp2* and *pfhrp3*. As a result, the two have also been shown to have much

diversity. However, *pfhrp3* has been shown to have lesser diversity (Jain *et al.*, 2014).

Since the discovery of *pfhrp2/pfhrp3* gene deletions in the late 1980s, many studies have been done in many different countries but Peru, India and Cambodia are the most studied countries. In the 2016 Global malaria information note of the WHO, *pfhrp2* deletions have been reported in four different countries in Africa with Ghana and Eritrea being the most recent and ongoing. Aside all the other procurement, storage and management of RDT factors, false negative results of RDTs have been attributed to these deletions in these reported countries. Reports by Amoah *et al* (2016) in Ghana showed the presence of the deletions of *pfhrp2* exon 2 in Ghana. Baker *et al* (2010) showed various genetic variations in the *pfhrp2* gene. Similar studies have also been done in Senegal, Mali and Eritrea (Baker *et al.*, 2005; Baker *et al.*, 2010).

Plasmodium lactate dehydrogenase gene

The *P. falciparum* gene which encodes for lactate dehydrogenase activity *pfLDH* is a single copy gene found on the 13th chromosome. This gene has no introns and are expressed as 1.6 kb mRNA in all the parasite's asexual blood stage. All malarial pLDH sequences share common epitopes (Hurdayal *et al.*, 2010) and therefore pLDH-based RDTs using mAbs against a common epitope can detect all human *Plasmodium* species, including mixed infections in circulating blood (Mayxay *et al.*, 2004). RDTs based on 'pan-malarial' LDH probably use mAbs against common epitopes.

The *pfLDH* shows high similarities between *P. falciparum* and vertebrates and bacteria (50 – 57 % similarity) *Escherichia coli* from which the gene was first synthesized but does not have the amino terminal extension found in all vertebrates. The *pfLDH* has most of its amino acid residues implicated in substrate and coenzyme binding and catalysis of other LDH are conserved in *pfLDH* (Bzik, Fox & Gonyer, 1993). This gene produces the pLDH glycolytic enzyme responsible for parasite glycolysis (Hurdayal, Achinolou, CHoveuax, Coetzer & Dean Golring, 2010).

The parasite and erythrocytic cells (human host) lack a complete citric acid cycle for mitochondrial ATP production and depend on anaerobic glucose metabolism, making pLDH an important enzyme for energy production in the parasite (Mouatcho & Goldring, 2013). This anaerobic activity of the enzyme is essential to the parasite and thus inhibition of this enzyme could kill the parasite (Sessions, Dewar, Clark & Holdbrook, 1997). The pLDH has peculiar properties like inability to inhibit other enzymes pyruvate concentrations and is very active with coenzyme 3-acetylpyridine (APAD) at 0.5 M lactate (Markwalter *et al.*, 2018).

***Plasmodium falciparum* Aldolase gene**

Fructose bisphosphate aldolase of *P. falciparum* is a gene with two exons and an intron (Knapp, Hundt & Kupper, 1990). The first exon codes for a single amino acid while the second exon codes for 368 amino acids. The gene is represented in the genome once which gets transcribed as a 2.4kb mRNA during

the blood stage of *P. falciparum*. The *pAldo* gene has a weight of 41 kDa with 61-67 % homology with eukaryotic aldolases. The *pAldo* gene produces a major enzyme involved in glycolysis of *Plasmodia* and is released into the blood during infection. The gene is localized in the cytoplasm of the parasite in an active and soluble form. The *P. falciparum* and *P. vivax* have one aldolase isoenzyme (Knapp *et al.*, 1990; Cloonan, Fischer, Cheng & Saul, 2001). The gene has a highly conserved region in all the *Plasmodia* making it the pan-specific for *Plasmodia* detection (Dzakah *et al.*, 2013; Kim *et al.*, 1998; Lee *et al.*, 2006).

Genetic Diversity

Knowing the DNA sequence variations and patterns for the *P. falciparum* surface antigens is very important in the ability to predict host immune responses. There are a lot of studies done to ascertain the fact that there have been multiple infections of genetically distinct strains of the *P. falciparum*. The WHO in accordance with these variations have put out a recommendation and stipulated a protocol which is used in the genotyping of all *P. falciparum* strains (WHO, 2007b).

The genetically diverse nature of the parasites provides proteins with multiple effective evasion and drug resistance mechanisms for the parasite. Merozoite surface proteins (*pfmsp1*, *pfmsp2* and *pfmsp3*) and glutamate rich protein (*glurp*) are commonly used markers for identifying *P. falciparum* in populations with diverse parasites genomes (Barry, Schultz, Buckee & Reeder, 2009).

CHAPTER THREE

MATERIALS AND METHODS

Study Design

The samples used for this study were randomly selected from archived blood cells and corresponding plasma samples collected during a multiple cross sectional community survey in January/February (Dry season) and July/August (Rainy season) of 2016.

Study Area

The parent study was conducted in Obom within the Ga South Municipality and Asutsuare within the Shai Osudoku District, both of the Greater Accra region of Ghana.

Greater Accra Region

The region has the smallest land area of 3,245 square kilometres, compared to the other 9 regions, which represents 1.4 % of the total land area of Ghana. It is the second most densely populated after region with a population of 4,010,054 (15.4 % of Ghana's total population) as at 2010 (Ghana Statistical Service (GSS), 2010). It is the region with the most widely internationally diverse ethnicity, however, the main natives are the Gas (GSS, 2014).

Ga South Municipality

The Ga South Municipality is a youthful community (Population and Housing Census, 2010) with a population of 411,377 persons representing 10.3 % of the region's population. (Ghana Statistical Service, 2014).

Obom

Domeabra Obom (Obom) is a rural farming community with about 22,360 children below 14 years (Amoah *et al.*, 2016), is found in the Ga South Municipality. (Ghana Statistical Service, 2014) (Figure 9). Malaria is perennial with the highest infections recorded in the rainy seasons from June to August. In 2014, it was estimated using microscopy that 35% of Obom clinic outpatient visits were *falciparum* malaria infections. According to some mapping studies done in the area, malaria parasite prevalence was estimated at 41 % during the peak transmission period in 2014 (Amoah *et al.*, 2016).



Figure 9: A map showing Obom. The red indicator shows the health center where the sampling took place. High transmission site of malaria.

Shai Osudoku District

Asutsuare in the Shai Osudoku District (Figure 10) representing a low transmission area of malaria. Shai Osudoku district is situated in the south-eastern part of Ghana in the Greater Accra Region. The district has a total land area of about 968.36 square kilometres, representing 29.84% land space of the Greater Accra Region (The Composite Budget of the Shai Osudoku District Assembly, 2015). The population of the district was 51,913 (GSS, 2010).



Figure 10: A map of Shai Osudoku District. Red arrow indicates Asutsuare, the study site.

Asutsuare

Asutsuare is a town located in Shai Osudoku district found in the Greater Accra of Ghana (Figure 10). The population consists of a small satellite village typically scattered along the Volta Lake with about 2000 people (GSS, 2010).

The population is mostly a fishing community. Malaria transmission has been reported to be low but perennial, and peaks slightly during and immediately after the major rainy seasons and is lowest during the dry seasons. The incidence of the disease has been reported to be 8.9 % with about 98 % of these infections caused by the *P. falciparum* (Adu *et al.*, 2016).

Sampling Procedure

Sample Size

This study was a pilot study which utilized 310 participants selected from the two study sites (161 from Obom and 149 from Asutsure). The number of samples used was also limited by the availability of paired plasma and blood cell pellets/dried filter paper blood blots

Inclusion Criteria

All samples with paired whole blood and corresponding plasma samples with available PfHRP2 RDT data were included.

Exclusion Criteria

Paired blood and plasma samples without RDT data or samples with RDT data but no matching plasma and blood cell pellet/dried blood spot samples were excluded from the study.

Ethical Approval/ Clearance

Ethical clearance was obtained from the Noguchi Memorial Institute for Medical Research (NMIMR) before the study was conducted. Approval was also sought from the chiefs and elders of the various communities. Before sample collection, the study was explained to the participants in comprehensible language after which they were given the chance to ask questions prior to being made to endorse age appropriate consent forms.

Materials

The Ethylene diamine tetra-acetic acid (EDTA) tubes and the Whatman filter paper (BD, UK) were used for the blood collection. Microscope slides (Fisher Scientific, USA) were used for processing the blood for microscopy. Malaria Ag (HRP2) CELISA Kit (Cellabs, Australia) for *P. falciparum* infection was used for the ELISA. The list of all primers used in the various PCR amplifications are contained in appendix V.

Reagents

The reagents for DNA extraction, Polymerase Chain Reaction and Gel electrophoresis were Zymo® Research Kit (Quick-gDNA™ Mini Prep), obtained from Inqaba Biotec West Africa Limited. Trizma base, Agarose powder, Ethidium bromide solution (10 mg/ml) (Sigma Aldrich, USA) , Calcium Chloride, Saponin, Chelex, Acetic acid, Sodium chloride and Absolute ethanol (Sigma, USA) were used. A 100 bp DNA ladder was obtained from Promega (U.S.A).

Methods

Genomic DNA (gDNA) samples were used to assess the presence of the *P. falciparum* histidine-rich protein II (*pfhrp2*) gene in DNA and *P. falciparum* histidine-rich protein II (PfHRP2) antigen in the plasma. The presence of the *P. falciparum* small subunit ribosomal RNA (*18s rRNA*) gene was used to ascertain the presence of *P. falciparum* in the samples. The integrity of the extracted DNA was confirmed by the presence of *pfmsp2* or *glurp* genes. Then the existence of *pfhrp2* and *pfhrp3* gene deletions were determined for samples that were *P. falciparum* and *pfmsp2/glurp* positive. All PCRs were repeated on samples that did not produce an amplicon. Samples that were consistently negative for any gene product were considered to be negative. The microscopy, hematocit (HB) and PfHRP2 RDT status of the samples were previously determined.

Genomic DNA Extraction

Deoxyribonucleic acid was extracted from frozen whole blood cells using the Quick-gDNA Mini Prep- Zymo Research Kit according to manufacturer's instructions and from dried blood blots using a modified Chelex method of extraction as described by Arez *et al.* (2000).

PCR amplification of isolates of *P. falciparum*

Small Subunit ribosomal RNA (18s rRNA)

Genomic DNA from the 3D7 strain of *P. falciparum* was used as positive control and double-distilled water used as negative control in all PCR the amplification reactions. Amplifications were carried out in 15 µL volumes

containing 1X Onetaq® PCR buffer (50 mM KCl, 10 mM Tris-HCl), 2.5 mM MgCl₂, 200 nM deoxynucleoside triphosphate mix (dNTPs) and 250 nM each of forward and reverse primers (rPU5 and rPlu6 for the primary PCR and rFAL1 (F) and rFAL2 (R) for nested PCR) and 1U of Onetaq™ DNA polymerase. This was done using the S1000™ Biorad Thermal cycler, Venendaal, Netherlands.

Four microliters (>20 ng) of each extracted gDNA was used as the template for the primary reaction and 2 µL of the primary reaction product was used as a template for the secondary reaction. The thermal cycle reaction conditions were 94 °C for 5 minutes (initial denaturation), followed by 30 cycles of 94 °C for 30 seconds, 58 °C (annealing temperature) for 1 minute and 68 °C for 1 minute. The final extension was performed at 68 °C for 5 minutes. .

Parasite Genotyping (*pfmsp2* and *glurp*)

Amplification of region II of the Glutamate rich protein 2 gene (*glurp*) and the central polymorphic region of the Merozoite rich protein 2 gene (*pfmsp2*) (3D7 and FC27 allelic families), were performed as described by Ayanful-Torgby *et al* (2017). PCR amplifications for the *pfmsp2* were multiplexed but GLURP was semi-nested.

All *pfmsp2* amplifications were carried out in a 15 µL reaction volume, containing 200 nM dNTP, 2 mM MgCl₂, 200 nM of each forward and reverse primer, and 1 U of Onetaq™ DNA polymerase (New England BioLAB, UK). Four microliters (>20 ng) of gDNA were used as the template for the primary reaction and 2 µL of the primary reaction product was used as a template for the secondary reactions. Two separate secondary reactions from Falk *et al* (2006),

both utilizing S1w primer paired with either the N5 primer (for the IC3D7 family) or M5 primer (for the FC27 family). The reaction cycling conditions were: initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of 94 °C for 30 seconds, 55 °C (annealing temperature) for 1 minute, and 68 °C for 1 minute. The final extension temperature was 68 °C for 5 minutes. The same conditions except annealing temperature of 58 °C were used for the secondary reaction.

The *glurp* amplification were carried out in 15 µL for the primary and 20 µL primary reactions volumes, containing 1X reaction buffer, 200 nM dNTP, 2.5 mM MgCl₂, 300 nM of each primer, and 1 U of Dream Taq DNA polymerase (Thermo Scientific, UK). Four microliters of gDNA was used as the template for the primary reaction and 0.5 µL of the primary reaction product was used a template for the secondary reaction using the outer and the primary primers. The reaction cycling conditions were initial denaturation at 94 °C for 2 minutes, followed by 30 cycles at 94 °C for 30 seconds, 58 °C (annealing temperatures) for 1 minute and 72 °C for 1 minute with a final extension at 72 °C for 5 minutes. The primary reaction conditions were same as the outer, however, the annealing temperature was set at 59 °C.

***Plasmodium falciparum* Histidine-Rich Protein Amplification**

All *pfhrp* amplification reactions were done in a 15 µL reaction volume. All *P. falciparum hrp* PCR amplifications were performed on Nexus Gradient Master Cycler (Eppendorf, Germany) and positive (laboratory strain parasites 3D7) and negative (double distilled water and Dd2) controls were used in all amplifications.

The *pfhrp2* exon 2 and *pfhrp3* exon 2 amplifications

The *pfhrp2* exon 2 PCR was done using a nested reaction. The 300 nM oligonucleotide primer sets used were 2.1 (F) and 2.2 (R) for the outer reaction while 2.3 (F) and 2.4 (R) for the primary reaction. 2.5 mM MgCl₂ was used for both primary and outer reactions. 5 µl of gDNA was used with 1 U working concentration per reaction of Onetaq™ Polymerase for both outer and primary reactions. The initial denaturation step for both reactions were 94 °C for 2 minutes, followed by 35 cycles 94 °C for 30 seconds, at 54.8 °C (annealing temperature) for 30 seconds for the primary PCR and 62 °C (annealing temperature) for secondary with extension temperature of 68 °C for 1 minute. The final extension temperature was 68 °C for 5 minutes. The *pfhrp3* amplifications were done using the same conditions as *pfhrp2* with the outer reaction oligonucleotide primers set 3.1 (F) and 3.2 (F) and primary primer set 3.3 (F) and 3.4 (R).

***Plasmodium falciparum* hrp1-2 gene amplification**

The *pfhrp1-2* PCR amplification was done using a nested reaction. The 300 nM oligonucleotide primer sets used were 2e12F and 2e12R for the reaction and 2 mM MgCl₂ for the reaction. Five microliters of gDNA was used with 1U working concentration per reaction of Onetaq™ Polymerase. The initial denaturation step for both reactions were 94 °C for 2 minutes, followed by 35 cycles 94 °C for 30 seconds, 57 °C (annealing temperature) for 30 seconds and

extension temperature of 68 °C for 1 minute. The final extension temperature was 68 °C for 5 minutes.

Agarose Gel Electrophoresis

Polymerase Chain Reaction amplicons were analysed. The gels were subjected to electrophoresis using 1X TAE as running buffer and ran at 100 V for 45 minutes to 1 hour. After electrophoresis, the gel was visualized and the image was captured with a Toyobo TM-20 UV Trans illuminator fitted with a camera or a Vilber Lourmat Gel Dock Systems® (Wielandstrasse, Germany).

Measurement of PfHRP2 antigen concentration

The PfHRP2 antigen levels were assessed using the malaria antigen CELISA kit (Cellabs, Sydney), which employs a sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturers instructions with slight modifications.

Recombinant HRP2 antigen (Standard/positive control antigen) was diluted three fold with Roswell Park Memorial Institute medium (RPMI) at a starting concentration of 110 ng/ml for 6 additional dilutions. 100 µL of the plasma samples together with the positive and negative controls (RPMI) were plated in duplicate onto a plate pre-coated with anti-*P. falciparum* monoclonal antibody. The plates were incubated in a humidified chamber for 1 hour at room temperature. This step was followed by extensive washing of the plate with PBS/Tween (0.1 %). 100 µL of the anti-*P. falciparum* conjugated antibody in a

1:200 dilution was added the plate and allowed to incubate in a humidified chamber for 1 hour at room temperature.

The conjugate was extensively washed off and 100 μL chromogen substrate at a 1:20 dilution was plated. The assay was kept in the dark for 15 minutes at room temperature after which a visible colour was noticed. 50 μL of 2 M sulfuric acid was added to stop the reaction. The plate was read spectrophotometrically at an optical density (OD) of 450 nm using Bio-Tek ELX 808TM plate reader.

Readings from the plasma samples were compared to the recombinant HRP2 curve to assess the HRP2 levels from the unknown samples. Optical density values that fell outside the linear range of the recombinant HRP2 were further diluted at 1:5 and repeated. Optical density values that remained off scale (low) upon further dilution were given a value corresponding to the least possible detection value.

A limit of 1.5 for defining a reading as positive was set for the absorbance value of the negative control plus 0.2 units, as the kit manufacturer suggested. A positive reading for PfHRP2 antigen was defined as an absorbance value above the cut-off level. A positive result indicate the presence of *P. falciparum*.

Statistical Analysis

Storage of data and generation of graphs were done using Microsoft Excel version 2013 and Graphpad prism version 6. ELISA analysis was done using Adamsel FPL b039 software by Ed Remarque (BPRC, Netherlands) which

converts OD values into concentrations and Graphpad prism version 6 statistical software. A standard curve was obtained from the recombinant HRP2 antigen standard using adamsel 309b. Analysis was done using t-test and One-way Anova's Dunn's post hoc test in Graphpad prism.

CHAPTER FOUR

RESULTS

Details of Study Participants

Although 310 samples were processed, the study focused on two sites, which are Asutsuare and Obom and the two major seasons in Ghana (Rainy and Dry seasons). One hundred and forty eight individual samples were used for the analysis, of which 60 (27 males & 33 females) were from Asutsuare and 88 (49 males & 39 females) from Obom. The mean age of the individuals in Asutsuare was found to be 21 years, ranging from 8 – 61 years while Obom recorded a mean age of 18 years, ranging from 6 – 45 years. During the period of study, Asutsuare and Obom recorded an average temperature of 31 and 35 °C respectively as seen in Table 1.

With respect to the haemoglobin (HB) concentration of the two sites, it was realized that the rainy and dry seasons of Asutsuare had mean HB of 12.47 g/dL and 12 g/dL respectively. Obom rainy season and dry season had mean values of 11.44 g/dL and 12.93 g/dL respectively. It can be seen that there is only a slight difference in Haemoglobin concentration between the two sites and the corresponding seasons (Table 1). There was significant difference between the means of HB from each season in each site after Kruskal-Wallis One-way analysis of variance ($p=0.0024$, $\alpha=0.05$). Comparison of parasite density between the two sites and their corresponding seasons showed that Asutsuare rainy and dry seasons had an average density of 32 p/ μ L and 40 p/ μ L respectively, while Obom

rainy and dry had 1600 p/μL and 221 p/μL respectively (Table 1). Parasite density was slightly statistically significant between the two sites using Kruskal-Wallis one-way analysis ($p=0.0491$, $\alpha=0.05$). Parasite density is the number of asexual parasites relative to a microliter of blood.

When RDT positivity was compared between the two study sites, using Kruskal-Wallis analysis, it was realized that there was a significant difference in RDT positivity between both sites. However, there was no difference between that of the seasons within each site (Figure 11).

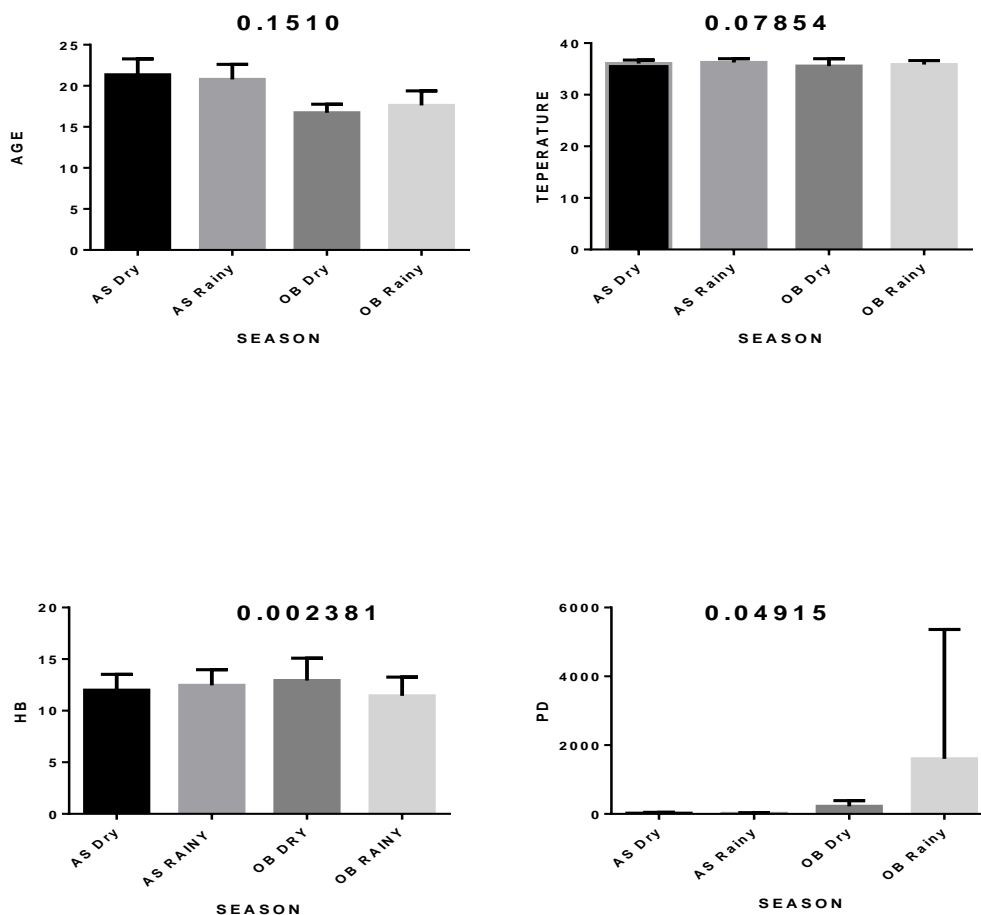


Figure 11: A Kruskal-Wallis comparison between seasons in Asutsuare (AS) and Obom (OB). p-values of each graph is indicated on top of it.

Parasite Identification

Using nested PCR (nPCR) for *P. falciparum* identification, 59.1 % of the 149 Asutsuare samples tested positive whereas 65.8 % of the 161 Obom samples tested positive (Figure 12). Excluding genotyping (*pfmsp2/glurp*) negative samples and using RDT positivity as a proxy for parasite prevalence, Asutsuare had a significantly lower (t-test: $p=0.0001$, $\alpha=0.05$) prevalence of *P. falciparum*, 1.7 % (1/60) while Obom had 53.4 % (47/88). *Plasmodium falciparum* infections prevalence difference between the study sites by microscopy (t test: $\alpha<0.05$ at 95 % CI - 0.4719 to 0.3059, $p=0.0001$) were 39.8% (35/88) in Obom and 3.3 % (2/60) in Asutsuare.

Table 1: Characteristics of the study population at enrolment

Parameter	Asutsuare		Obom	
	Rainy season	Dry season	Rainy season	Dry season
Sex				
Male N	10	17	19	30
Female N	8	25	9	30
Age (Years)				
Mean	21	21	18	17
SEM	1.834	1.948	1.782	1.040
Range	(11-35)	(8-61)	(10-45)	(6-44)
N	18	42	28	60
Temperature °C				
Mean	36.2	36	35.8	35.5
SEM	0.1751	0.1050	0.1492	0.1863
Range	(33.8-37.2)	(34.30-37.6)	(33.8-37.2)	(30.20- 37.5)
N	18	42	28	60
Haemoglobin (g/dL) *				
Mean	12.47	12	11.44	12.93
SEM	1.512	1.528	1.814	2.164

Range	(9.7-15.7)	(6.6-14.5)	(7.9-14.9)	(6.0- 17.7)
N	18	42	28	60
Parasite density/μL of blood *				
Mean	32	40	1600	221
SEM	0	0	3640	166
Range	(0-32)	(0-40)	(160-13970)	(32-555)
N	18	42	28	60
n	1	1	17	15
HRP2 (RDT) % *				
Positive	5.56	0	53.57	53.33
N	18	42	28	60

* - statistically significant by t-test analysis, n- number positive , SEM- standard deviation, N- population tested

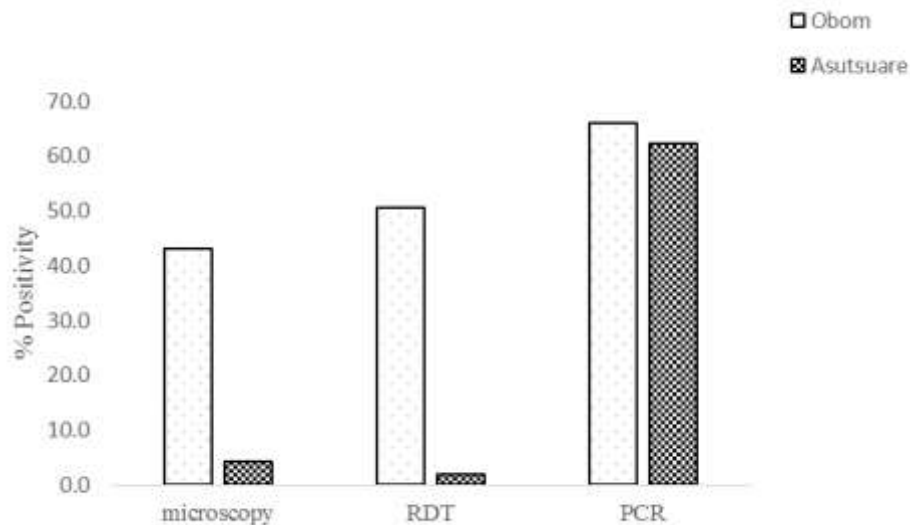


Figure 12: Comparison of RDT, microscopy and PCR between the study sites samples. PCR used was based on the pre-inclusion population of Obom (161) and Asutsuare (149) whereas the RDT and microscopy were based on the genotyped (*pfmsp2/glurp*) positive samples

For PCR amplification of *18s rRNA*, a band size of 205 bp was expected for each amplicon from both study sites and an image was recorded in Figure 13.

This showed prominent bands for 1, 8, 9 and 10. Samples 11 and 12 showed faint bands hence were also counted as positive for *18s rRNA* gene amplification.

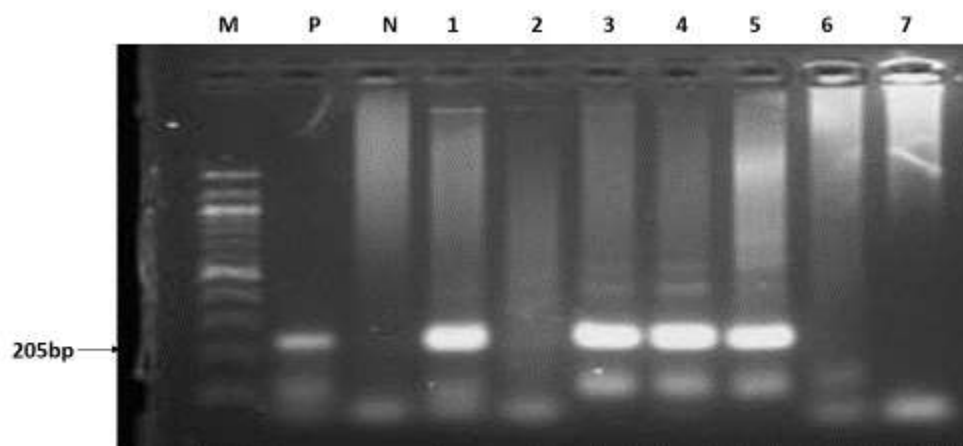


Figure 13: A gel image after PCR amplification of *18s rRNA* speciation for *P. falciparum*. Negative control (N), 3D7- Positive control (P), 1-7: 7 different samples

***Plasmodium falciparum* speciation in Obom and Asutsuare**

Among the *18s rRNA* positive samples within the study sites, the percentage positives of *Pfmsp2/glurp* genotypes were 75.0 (60/88) and 83.02 (88/106) for Asutsuare and Obom respectively. Gels images showing amplicons of different band sizes for 3D7 allelic regions of the *Pfmsp2* has been presented in Figure 14.

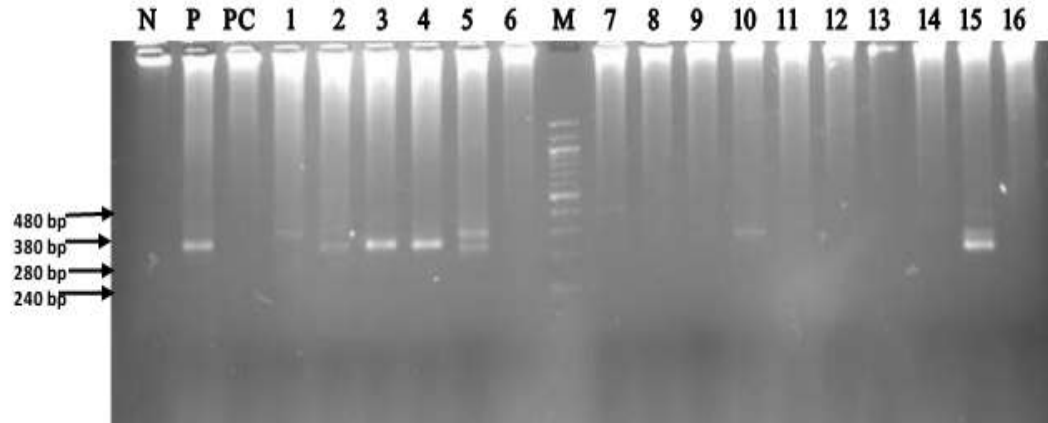


Figure 14: A gel image of *Pfmsp2* (Primers: M2-OF, M2-OR, S1fw, M5rev and N5rev) showing different band sizes. PCR amplification of *Pfmsp2* showing different band sizes where 3D7 and K1 are positive controls, N; H₂O (negative control) representing double distilled DNase free water, M is 100 bp DNA ladder and 1-16: 16 different gDNA samples

Out of the genotyping positive samples from Asutsuare, 96.6 % were RDT and microscopy negative. Also, 3.4 % were RDT negative but microscopy positive. One sample was RDT positive and microscopy negative representing 100 percent for the positive RDTs and none was recorded positive for microscopy and RDT. Obom had 78 % which were RDT and microscopy negative. Obom also had 22 % which were microscopy positive but RDT negative. A total of 44.7 % were RDT positive but microscopy negative for Asutsuare while 55.3 % were RDT positive but microscopy negative for Obom (Table 2).

Table 2: Prevalence of genotyping positive samples for RDT and microscopy

Study site	RDT	Microscopy	
		Negative	Positive
Obom	Negative (41)	78.0	22.0
	Positive (47)	44.7	55.3
Asutsuare	Positive (1)	100.0	0.0
	Negative (59)	96.6	3.4

(n) represents number of samples tested

The *pfhrp2* and *pfhrp3* percentage positives

All gDNA samples that tested positive for both *18s rRNA* species specific PCR as well as *Pfmsp2/glurp* (genotyping) were considered to be of good quality, for which *pfhrp2* and *pfhrp3* amplifications can be performed on (WHO 2010). The PCR results for all the *hrp2* genes involved in this study have been reported in Figure 13. The rainy season of Asutsuare recorded 66.7 %, 11.1 and 77.8 out of 18 samples to be positive for *pfhrp1-2*, *pfhrp2* and *pfhrp3* respectively. However, the dry season out of 60 samples recorded 64.3 %, 14.3 % and 66.7% of *pfhrp1-2*, *pfhrp2* and *pfhrp3* respectively. The rainy season of Obom recorded 78.6 %, 60.7 % and 53.6 % out of 28 samples to be positive for *pfhrp1-2*, *pfhrp2* and *pfhrp3* respectively. The dry season, however, out of 60 samples recorded 88.3 %, 60.0 % and 45.0 % of *pfhrp1-2*, *pfhrp2* and *pfhrp3* respectively (Figure 15).

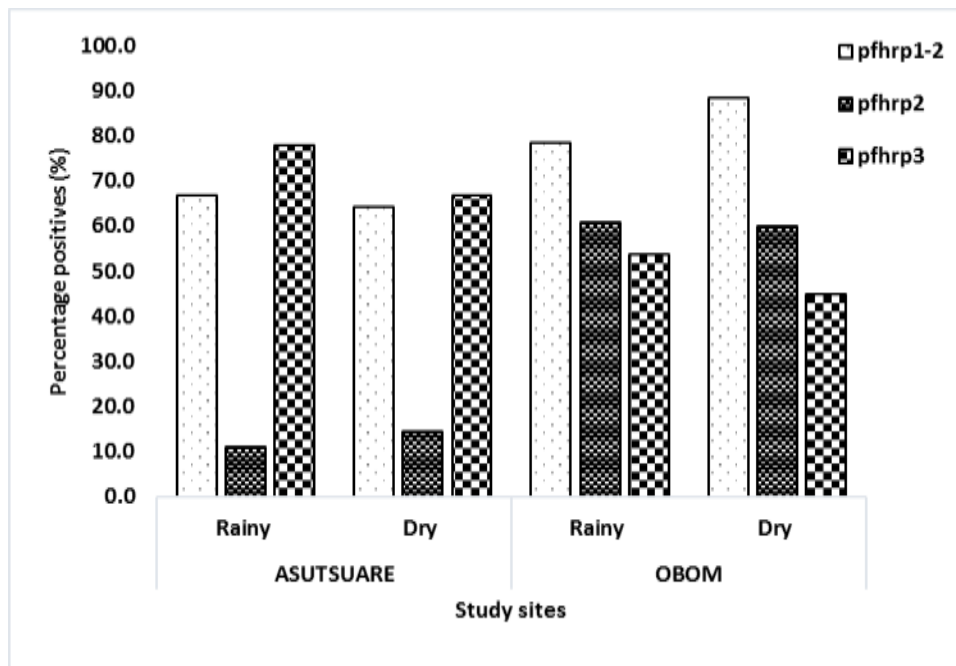


Figure 15: Comparison of *pfhrp1-2*, *pfhrp2* and *pfhrp3* PCR. This compares rainy season and dry season distribution of *pfhrp* genes between Obom and Asutsuare..

The exon 2 of the *pfhrp2* was amplified using the primers described in Appendix IV. Figure 16 shows PCR result of the amplification of 305 nucleotides of *pfhrp2* exon 2.

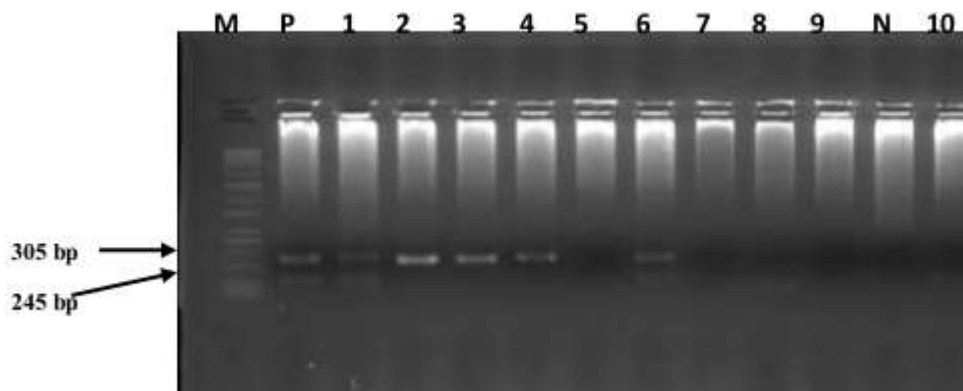


Figure 16: A gel image of *pfhrp2* exon 2 amplification showing two different bands. PCR amplifications of a (*pfhrp2*) exon 2. 100 bp marker (M), Negative control (N) was DNase free double distilled water, Positive control (P) was 3D7 parasite culture DNA and 1-10 are 10 different samples

The *pfhrp3* amplifications yielded PCR products of 205 nucleotides which have been described in Figure 17. Sample 11 had more than one amplicon showing presence of multiple different *pfhrp3* genes.

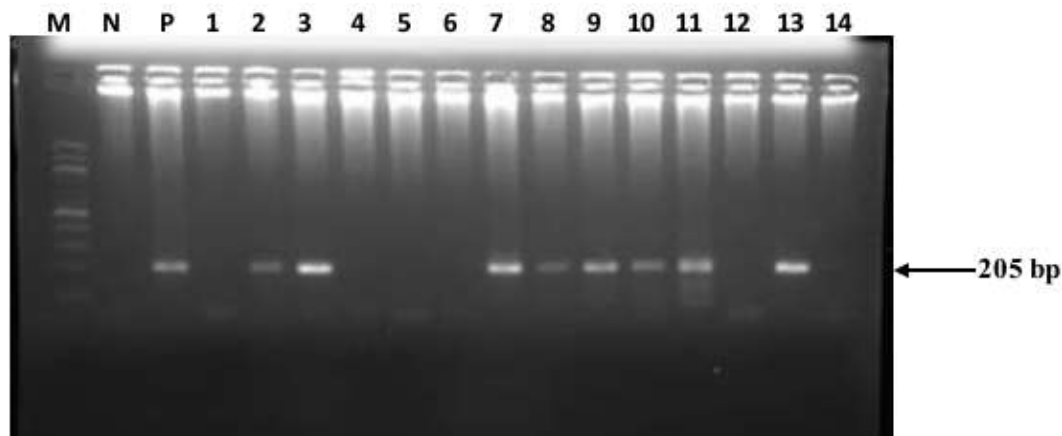


Figure 17: A gel image of *pfhrp3* exon 2. An image of *pfhrp3* gel showing the expected band size. Where P: 3D7 Positive control, N: negative control, 1-14: samples

The *pfhrp1-2* was positive for almost all the genotyping positive samples in Asutsuare and Obom. The exons was however amplified with introns in them. This amplified 420 bp and were viewed using UV light in a gel documentation system. A gel image of *pfhrp1-2* has been used to represent positivity in Figure 18.

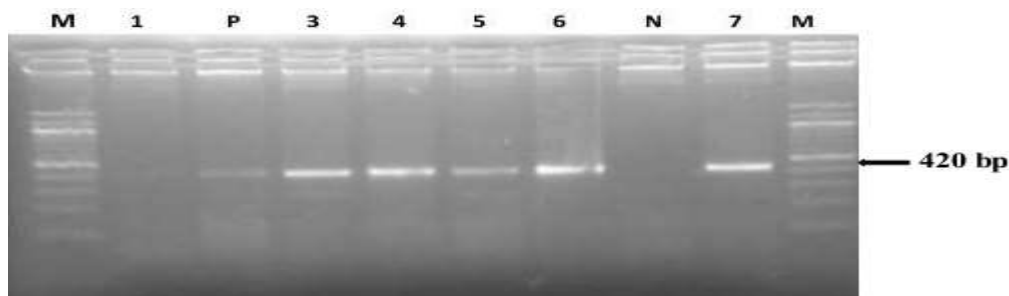


Figure 18: A gel image of *pfhrp1-2* showing bands for different samples. A PCR amplification of *pfhrp1-2* gene. M (100 bp DNA ladder), N (ddH₂O-negative control), 1 – 7 (Samples) and P (3D7-positive control).

Analysis of Deletions of *pfhrp2* and *pfhrp3*

Samples were considered positive for *pfhrp2* if any/both of the genes for *pfhrp2* and *pfhrp1-2* was positive after PCR. The total number of positives and negatives for *pfhrp2* and *pfhrp3* have been summarized in percentages. Also, the total number of negatives representing deletions of these genes are represented in Figure 19. Double negatives for *pfhrp2/pfhrp3* depicted deletions for the *pfhrp2* and *pfhrp1-2* genes and *pfhrp3* exon 2 gene. Therefore, Obom had 1.7 % *pfhrp2/pfhrp3* deletions while Asutsuare had 4.8 % both in the dry season. However, these deletions were all recorded in the dry (low malaria) season with no deletions occurring during the rainy (peak malaria) season. This showed higher deletions in low transmission area, Asutsuare during the low malaria season than in the high transmission area, Obom during the low malaria season (Table 3).

Table 3: Analysis of *pfhrp2* and *pfhrp3* deletions

Seasons	Study sites	<i>pfhrp2</i>		<i>pfhrp3</i>		<i>pfhrp2/pfhrp3</i>	
		Pos (%)	Neg (%)	Pos (%)	Neg (%)	Double Pos (%)	Double Neg (%)
Rainy	Asutsuare (18)	72.2	27.8	77.8	22.2	50	0
	Obom (28)	100	0	53.6	46.4	53.6	0
Dry	Asutsuare (42)	71.4	28.6	66.7	33.3	42.9	4.8
	Obom (60)	95	5	45	55	35	1.7

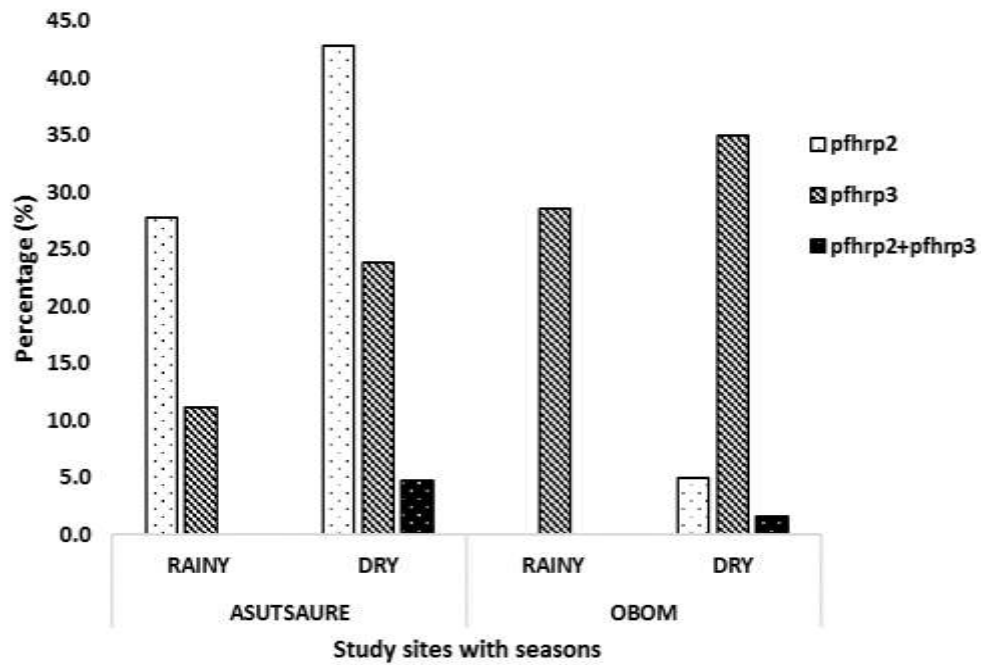


Figure 19: Deletions of individual genes. The chart shows the percentage of deletions of *pfhrp2* (this includes *pfhrp2* and *pfhrp1-2*), *pfhrp3* and both *pfhrp2* and *pfhrp3*

There were 15 samples from Asutsuare which were *pfhrp1-2* and *pfhrp2* negative but *pfhrp3* positive and 2 samples from Obom. Thus, they are seen to substitute for the *pfhrp2* deletions. In Obom, all the *pfhrp3* substitution was found in the dry season with none in the rainy season. Asutsuare had 33.3 % of the 15 samples in rainy season but 66.7 % in dry season (Table 4.)

Table 4: *pfhrp3* positive but *pfhrp1-2* and *pfhrp2* negative

Sites	Season	<i>Pfhrp3</i> +ve / <i>pfhrp2</i> -ve (%)
Obom (2)	Rainy	0
	Dry	100
Asutsuare (15)	Rainy	33.3
	Dry	66.7

Serological Confirmation and Quantitation of the PfHRP2 Antigen by ELISA

A total of 92 plasma samples from Obom and 49 samples from Asutsuare that were randomly selected and used for the PfHRP2 CELISA sandwich ELISA.

Most PfHRP2 RDT negative samples were also negative for ELISA in Asutsuare whilst Obom had most PfHRP2 RDT negatives being ELISA positive. A higher number of RDT and *pfhrp2* PCR positives were identified in Obom relative to Asutsuare (Figure 20).

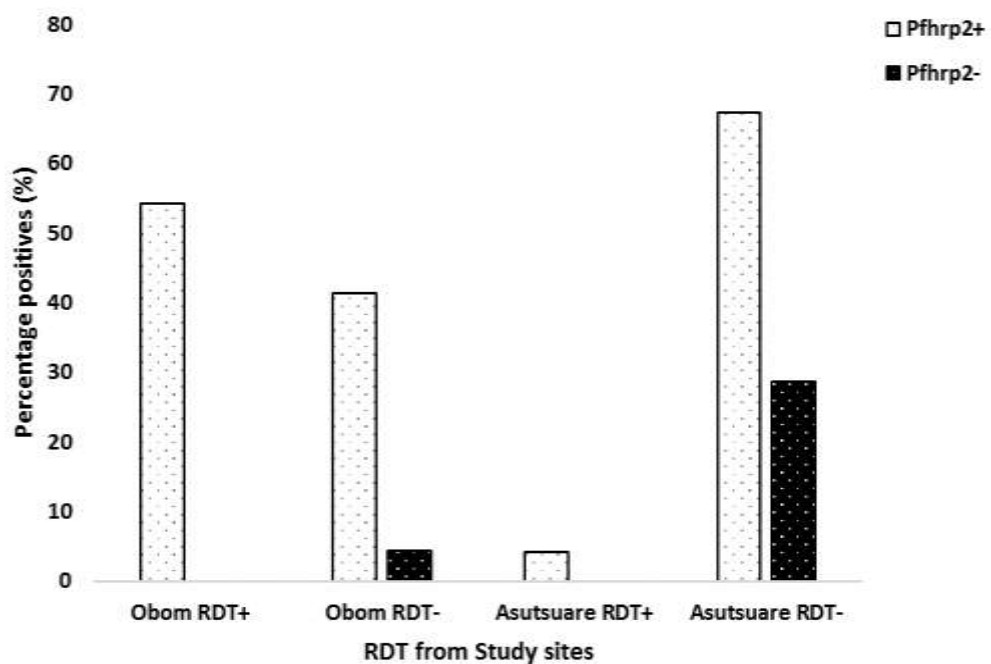


Figure 20: Distribution of ELISA samples according to their RDT results. Samples that were positive for RDT which were also positive for the ELISA

The mean concentrations of PfHRP2 in Obom and Asutsuare samples according to their microscopy and RDT results are summarized in Table 5. There

were no samples which was positive for both RDT and microscopy in Asutsuare. However, there were 26 samples of such in Obom.

Out of the 92 samples from Obom, 28.3 % of them were positive for both microscopy and RDT and had an average PfHRP2 antigen concentration of 214.8 ng/ml. Samples which were negative for both microscopy and RDT were 32.6 % with an average PfHRP2 concentration of 3.2 ng/ml. Samples which were positive for RDT but negative for microscopy were 21.7 % and had average PfHRP2 concentration of 181.1 ng/ml. Those that were positive for microscopy but negative for RDT were 15.2 % with 21.3 ng/ml PfHRP2 concentrations. None of the 49 samples from Asutsuare was positive for both RDT and microscopy. A large number, 83.7 % of the Asutsuare samples were negative for both RDT and microscopy. This recorded a very low average PfHRP2 concentration of 8.4 ng/ml. Samples from Asutsuare that were positive for RDT but negative for microscopy were 4.8 % and had an average PfHRP2 concentration of 550.0 ng/ml. Samples that were positive for only microscopy were 12.2 % with an average PfHRP2 concentration of 7.4 ng/ml (Table 5).

Table 5: Mean PfHRP2 antigen concentrations in relation to microscopy and PfHRP2 RDT results.

Sites	Tests	SD	PfHRP2 antigens (ng/ml)	Number of Samples
	RDT+/Microscopy +ve	3930.45304	5584.5	26

Obom	RDT-ve/Microscopy –	18.9504617	3.2	30
	ve			
	Only RDT +ve	113.914902	181.1	20
	Only Microscopy +ve	5.86898628	21.3	13
	RDT+ve/Microscopy	0	0.0	0
	+ve			
Asutsuare	RDT-ve/Microscopy –	23.0516811	8.4	41
	ve			
	Only RDT +ve	387.494516	550.0	2
	Only Microscopy +ve	0.98994949	7.4	6

+ve = positive ; -ve= negative,

The highest PfHRP2 concentration in Asutsuare was 1099.97 ng/ml while the lowest recorded was 0.085 ng/ml. In Obom the highest PfHRP2 concentration was 2001.95 ng/ml and 0.005 ng/ml was the lowest. About 78% of the Asutsuare samples had low concentrations while it was 23.9 % in Obom

CHAPTER FIVE

DISCUSSION

False RDT negative results are very derogatory to malaria control (Kozycki *et al.*, 2017; Ranadive *et al.*, 2017) as the main diagnosis practices in most health facilities confirm RDT positive tests by microscopy (Aregawi, Cibulskis, Otten & Williams, 2009). This leaves all RDT negative cases untreated for malaria. One of the major causes of false negative PfHRP2 RDT is the presence of deletions in *pfhrp2* and *pfhrp3* genes which also consequently influence test kit sensitivity (Msellem *et al.*, 2009; Maltha *et al.*, 2014; Ranadive *et al.*, 2017). Seasonal changes play an important role in malaria transmission. This is mainly due to the survival rate of the vector during these climatic conditions. The vectors tend to multiply more in the rainy season than in the dry season because stagnant waters become rampant for easy breeding of the mosquito. Rainy and dry seasons in Ghana therefore determine the rate of the disease's infections.

This study sought to compare the use of microscopy, RDT and PCR to determine the clinical efficiency of malaria among the two communities and seasons and to determine *pfhrp2* gene deletions in *P. falciparum* parasites circulating in the two transmission settings (Figure 11). In most field and epidemiological studies, microscopy and RDT are mainly used to determine malaria prevalence (Azikiwe *et al.*, 2012), whereas, PCR or ELISA are used to determine prevalence on policy based programmes of National Malaria Control Programmes (Msellem *et al.*, 2009).

Comparing the use of RDTs and PCR in detecting the presence of the *Plasmodium* parasites in a low transmission area in Zambia, Laban *et al* (2015) recorded very low parasitaemia in many parts with few gene deletions in the area. However, this study showed that the low transmission area had more gene deletions than the high transmission area and thus contradicts their earlier studies. The low transmission area in this study have been known to produce very few malaria positives and also hardly produce any positives during the off-peak malaria seasons. This may give more room for the parasite's mutations, as a result, finding a way to escape been seen. Gene deletion may be the most appropriate mutation for the parasite.

Determination of the prevalence of the *P. falciparum* parasite through the use of RDTs, microscopy and PCR gave different results. Most studies which PCR/ microscopy and PCR/RDTs showed high differences (Mogeni *et al*, 2017; Boonman, Christensen, Suwanarusk, Price, Russell and Lek-Uthai, 2007). Malaria microscopy and RDTs in both study sites followed the similar results with high difference between the microscopy in Obom (High transmission zone) and that of Asutsuare (Low transmission zone) as shown in Figure 11. The main reason why these differences are so wide have been attributed to the detection limits of microscopy set at ≥ 50 parasites/ μL (Azikiwe *et al.*, 2012) and PCR detection limit of as low as 10 parasites/ μL (Wangai *et al.*, 2011).

The PfHRP2 RDT and microscopy showed high difference between the two sites in percentage of positives. However, in PCR, Obom showed slightly higher percentage positives than Asutsuare (Figure 11). This showed high

positivity in the area but the PfHRP2 iRBCs were in very low quantities and thus could not easily be captured by either microscopy or RDT (Assumpcao, 2016, Unpublished). This confirms the fact that there must be PCR confirmation of prevalence before it is considered as low or high (Fançony, Sebastião, Pires, Gamboa & Nery, 2013). Also, the RDTs should be made more sensitive to capture very low amount of proteins in the blood.

The prevalence of *P. falciparum* infections in Obom through the use of PCR showed higher percentage positives in Obom compared to Asutsuare. Infection prevalence using *pfmsp2* or *glurp* specific primers in PCR was lower in Asutsuare than Obom. Obom showed higher *pfhrp2* and *pfhrp3* PCR gene amplifications but lower amplifications in Asutsuare as shown in Figure 14. This implied that *P. falciparum* infection is higher in the Asutsuare (low transmission zone) than earlier reported (Adu *et al.*, 2016). This is the reason why WHO *P. falciparum* genotyping protocol requires that either *pfmsp1*, *pfmsp2* or *pfmsp3* and/or *glurp* be used to determine the quality of the parasite DNA before doing the *pfhrp2* PCR (WHO, 2010).

This study recorded some deletions of *pfhrp2* and deletions of *pfhrp3* (Table 3). This confirms the presence of *pfhrp2/pfhrp3* gene deletions in Ghana as reported earlier by Amoah *et al* (2016). However, gene deletions were more prevalent in the low malaria season than in the peak malaria season (Figure18). The study by Amoah *et al* (2016) reported on only the exon 2 of the *pfhrp2* and *pfhrp3* but this present study reports on *pfhrp1-2*, *pfhrp2* and *pfhrp3*. *Plasmodium falciparum* HRP2 RDTs have been used to improve malaria diagnosis in many

countries but false negative results in Ghana has made malaria difficult to eradicate (Msellem *et al.*, 2009; Koita *et al.*, 2012). Since Ghana is known to be an endemic country with high prevalence rate in the peak seasons, treatment may often be given even when RDTs test negative results are recorded. Thus, parasites may not get enough chances for mutations. In Eritrea, deletions were highly reported during peak malaria seasons (Berhane *et al.*, 2018). However, this present study showed that deletions were more prevalent during the low malaria seasons (off-peak season) in both study sites.

In Yemen, isolates with very low parasitaemia of 98 parasites/ μ l which were diluted with a 100 μ L O⁺ blood were still positive through the use of RDT but concentrations of 65 parasites/ μ l recorded negative (Atroosh *et al.*, 2015). In this study, a similar finding was obtained when parasite density at Asutsuare was lower than 65 parasites/ μ L confirming the results of the current study that most samples from the low transmission area were negative through the use of RDT (Table 1).

In Democratic Republic of Congo, deletions of *pfhrp2* among children under 5 years were few at 6.4 % (Parr *et al.*, 2016), similar to what this study revealed in Obom at 1.7 % (Table 4). Parr *et al.* (2016) found very few *pfhrp2/pfhrp3* deletions between the Kinshasa and Kivu provinces though both were earlier reported to be high transmission areas. However, this was attributed to the fact that RDTs had earlier been introduced into the regions and had had the highly prevalence reduced as a result of early treatment. This was done in comparison to other places where RDTs were lately introduced (Parr *et al.*). RDT

negative samples were becoming high in the area causing a reduction in prevalence set for the two provinces. This could mean that gene deletions contributed to the RDT negative rates in these regions just as earlier studies showed high prevalence and low prevalence lately in Asutsuare (Adut *et al.*, 2016; Attu & Adjei, 2018).

The *pfhrp2* and *pfhrp3* exon 2 deletions have mostly been reported to be many in different areas (Amoah *et al.*, 2016; Kumar *et al.*, 2013; Okoth *et al.*, 2015; Viana Rahid *et al.*, 2017). However, *pfhrp1-2* deletions have not been reported in earlier studies in Ghana. In this study, it was discovered that some *pfhrp2* genes had the exon 1 deleted. These deletions were prominent in Asutsuare than in Obom just as it was for the *pfhrp2* and *pfhrp3* deletions (Figure 18), so this could affect the concentrations of PfHRP2 proteins in the samples.

Laban *et al* (2015), showed that there were few gene deletions and low false positives in the low transmission areas in the region of study. Thus, this study came out with a similar finding of low false negatives of 4.8 % in Asutsuare and 1.7 % false negatives in Obom (Table 3). All deletions were reported in the dry season (low malaria season). This indicates that studies in high transmission areas may not necessarily show high percentage of false negatives if there have not been much mutations.

Okoth *et al* (2015) found out that PfHRP2 RDT could still be useful in Guyana (French Guiana) since 249 samples collected between 2009 and 2011 showed no *pfhrp2/pfhrp3* deletions. In Suriname on the other hand, few

pfhrp2/pfhrp3 deletions were recorded raising concerns for the use of PfHRP2 RDTs in the country (Okoth *et al.*, 2015). It was reported that most migrant miners rotated between the closer countries and so gene deletions could be seen in later years.

Despite the prevalence of 42% in Guyana, there was no *pfhrp2/pfhrp3* deletions in the country (Okoth *et al.*, 2015). The findings of Okoth and the colleagues support the results of the present study since the high transmission area (Obom) had low *pfhrp2/pfhrp3* deletions of 1.7 % as compared to 53.4 % of RDTs positive and 39.8 % microscopy positives. The use of PfHRP2 RDTs should therefore not be the major diagnostic tool within the lower transmission areas. However, they could still be of help in malaria diagnosis in high transmission areas.

In this study, it was found out that *pfhrp2* and *pfhrp3* deletions did exist in these study sites, however, some of the *pfhrp2* deletions were compensated for by the *pfhrp3* gene (Table 4). Though there was a 1.7 % total *pfhrp2/pfhrp3* gene deletions from Obom, another 2.3 % of the samples was *pfhrp2* gene deleted but was compensated for by the *pfhrp3* gene in RDT (Table 3). Asutsuare also had 27.8 % *pfhrp2* deletion in the dry season and 42 % in the rainy season and 4.8 % *pfhrp2/pfhrp3* deletions in the dry season. Nearly 24 % showing *pfhrp3* positive but *pfhrp2* negative. This confirms earlier reports that *pfhrp3* gene may compensates for *pfhrp2* when the later is deleted (Gamboa *et al.*, 2010; Pain *et al.*, 2014; Okoth *et al.*, 2015). This possibly accounted for the positivity of the RDT knowing that when the *pfhrp2* gene is deleted, *pfhrp3* can substitute for it.

This study showed that the total *pfhrp2/pfhrp3* gene deletion was higher in the low transmission area, Asutsuare than in the high transmission area, Obom. This is contrary to what was found in Columbia by Abeku *et al* (2008). It was as well revealed that during low malaria seasons there are more gene deletions than during high malaria seasons. This implies that gene deletions may not necessarily be due to high transmission but probably mutations of parasites in the area. There may also be high transmission but low parasite mutations whereas low transmission may have high mutations due to migration of individuals (Okoth *et al.*, 2015; Laban *et al.*, 2015).

Fourteen of the plasma samples from Asutsuare were negative for *pfhrp2* through PCR. One sample showed positive for ELISA and this was among the four samples which were microscopy positive. Though PCR was negative for three out of four microscopy positives, one sample was positive for ELISA. This one ELISA positive sample recorded PfHRP2 concentration of 45 ng/ml showing higher concentrations in the plasma (Table 5). This could be that the parasite had been with the individual for a longer time while the other three could be early stage infection or lower parasitaemia (Pava, Echevery, Diaz & Murillo, 2010). As a result, the later group of samples may not easily be detected by either RDT or ELISA. Ten samples from the low transmission area tested negative for *pfhrp2* through PCR, RDT and ELISA. This shows that the area has low infection rate since 20.4% were confirmed negative by ELISA just as other studies have used ELISA to confirm positivity in epidemiological studies (Dondorp *et al.*, 2005; Martin, Rajasekariah, Awinda, Waitumbi & Kifude, 2009)

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

The study was conducted mainly to determine *pfhrp2* and *pfhrp3* gene deletions in *P. falciparum* parasites circulating in Obom (high transmission area) and Asutsuare (low transmission area) in the Greater Accra Region of Ghana. One-hundred and sixty two participants were selected from each site based on the availability of samples' plasma. Ethical clearance was obtained from NMIMR and GHS ethical review committee before the study was conducted. Approval was also sought from the administrators of the various health facilities before sample collection.

Samples from both communities which have been confirmed by microscopy and/or RDTs to be malaria positive or malaria negative were randomly selected and used to assess the presence of the PfHRP2 antigen. The presence of the *18s rRNA P. falciparum* gene was used to ascertain the presence of the *P. falciparum* species. The percentage positives for *pfmsp2/glurp* and *pfhrp2* were also determined on the same samples to determine the *P. falciparum* genotypes and *pfhrp2* deletions respectively. Deletions of *pfhrp2* and *pfhrp3* genes were identified in both settings.

Conclusions

Based on the results obtained in the study, the following conclusions were drawn.

1. There were differences in the clinical efficiency of PCR, RDT and microscopy in malaria parasites detection between the low transmission area, Asutsuare and the high transmission area, Obom.
2. Deletions of *pfhrp2* and *pfhrp3* genes were identified and confirmed in the two study sites during the dry season which is low malaria transmission season.
3. The levels of PfHRP2 proteins in the higher transmission area, Obom was higher than in the low transmission area, Asutsuare. RDTs are generally useful in malaria endemic regions but is not appropriate for areas of low transmission.

Recommendations

1. Further studies should be done nationwide among symptomatic individuals in order to ascertain the prevalence of *pfhrp2/pfhrp3* deletions and which types of deleted *pfhrp2* and *pfhrp3* genes are present in different areas.
2. Gene sequencing should be done for all alleles present in the areas to be able to determine the various wild types of *pfhrp2* and *pfhrp3* deletants in Ghana.

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APPENDIX I

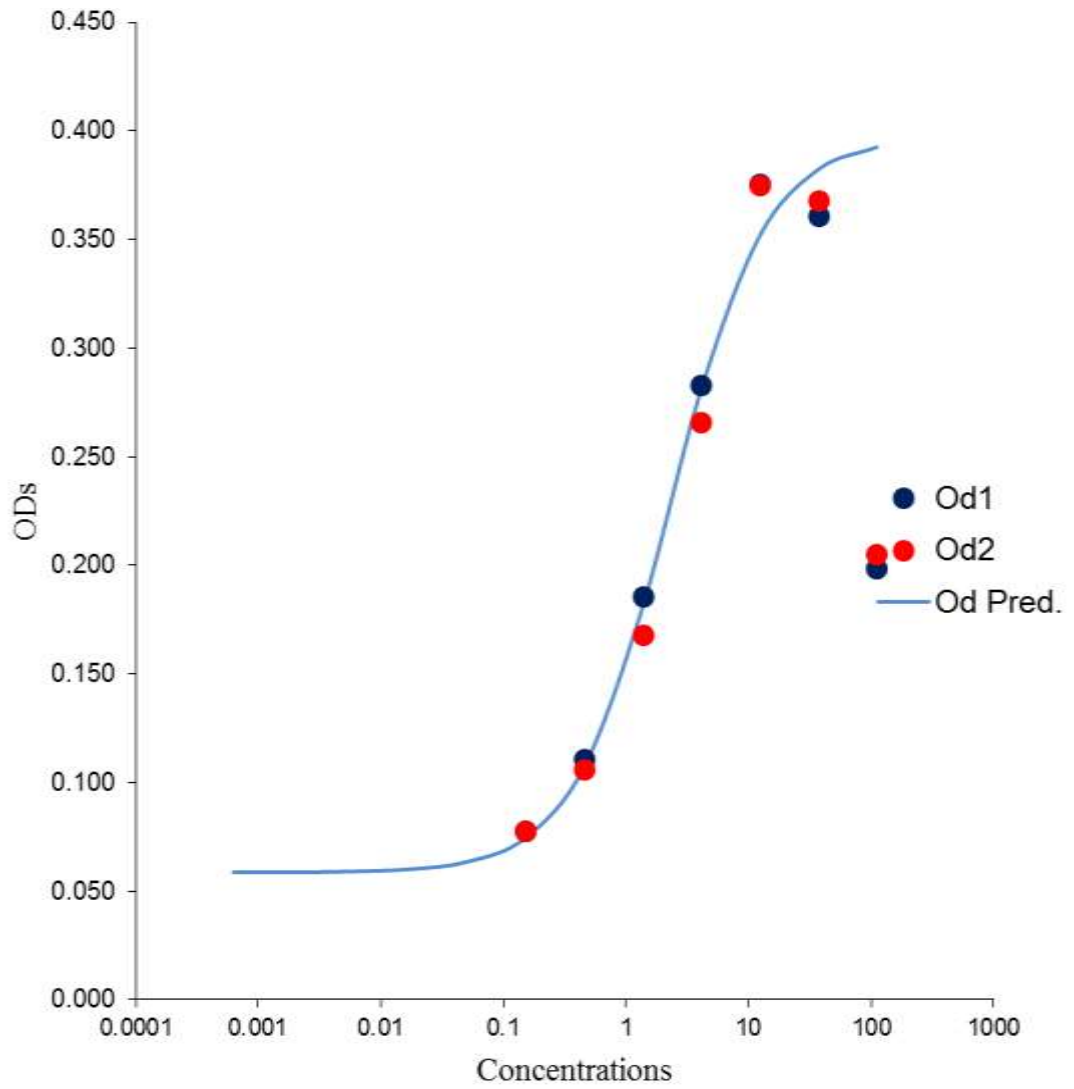
Supplementary Table 1: Speciation, Genotyping *pfmsp2* and *glurp* positive with *hrp2* results

sample ID	18s rRNA	PFMSP2	GLURP	hrp2 1-2	hrp2 Ex2	hrp3 Ex2
AS 14	+	-	+	-	-	-
AS 23	+	-	+	+	-	+
AS 37	+	-	+	-	-	-
AS 41	+	-	+	+	-	-
AS 44	+	-	+	+	-	+
AS 45	+	-	+	+	-	-
AS 49	+	-	+	-	-	+
AS 53	+	-	+	+	-	-
AS 74	+	-	+	-	-	+
AS 78	+	-	+	-	+	-
AS 81	+	-	+	+	-	-
AS 84	+	-	+	-	-	+
AS 85	+	-	+	-	+	+
AS 89	+	-	+	+	-	+
AS 95	+	-	+	-	-	+
AS 100	+	-	+	-	-	+
AS 110	+	-	+	-	-	+
AS 133	+	-	+	+	-	+
AS 185	+	-	+	+	-	-
AS 196	+	-	+	+	-	+
AS 215	+	-	+	+	+	-
AS 235	+	-	+	+	-	-
AS 254	+	-	+	-	-	+
AS 260	+	-	+	-	-	+
AS 262	+	-	+	-	-	+
AS 266	+	-	+	-	-	+

AS 269	+	-	+	+	-	+
AS 260	+	-	+	-	-	-
AS 263	+	+	+	+	-	-
AS 264	+	-	+	-	-	+
AS 267	+	+	-	-	-	+
AS 268	+	-	+	+	+	+
AS 281	+	-	+	-	+	+
AS 282	+	-	+	+	-	+
AS 284	+	-	+	-	+	+
AS 285	+	-	+	+	-	+
AS 286	+	-	+	+	+	+
AS 287	+	-	+	+	-	+
AS 288	+	-	+	+	-	+
AS 290	+	+	-	+	-	+
AS 291	+	-	+	-	-	+
AS 292	+	-	+	+	+	+
AS 294	+	-	+	-	-	+
AS 307	+	+	-	+	-	+
AS 308	+	-	+	+	-	+
AS 309	+	-	+	+	-	+
AS 310	+	-	+	-	-	+
AS 315	+	-	+	+	-	+
AS 317	+	-	+	+	-	-
AS 318	+	+	-	+	-	+
AS 320	+	+	+	+	-	+
AS 325	+	-	+	+	-	+
AS 342	+	-	+	+	-	+
AS 344	+	-	+	+	-	+
AS 350	+	-	+	+	-	-
AS 353	+	-	+	+	-	-
AS 402	+	-	+	+	-	+
AS 408	+	+	-	+	-	+

AS 424	+	+	-	+	-	-
AS 477	+	-	+	-	-	+
AS 500	+	-	+	-	-	+
AS 521	+	-	+	+	-	+
AS 530	+	-	+	+	-	-
AS 537	+	-	+	+	-	+
AS 550	+	-	+	+	-	+
AS 562	+	+	+	+	+	+
AS B	+	-	+	+	-	+
AS C	+	-	+	-	+	-
AS E	+	-	+	+	-	+
AS F	+	-	+	+	+	+
AS K	+	-	+	+	-	+
AS Q	+	-	+	+	-	-
AS S	+	-	+	-	+	-
TOTAL	73	9	67	47	12	53

APPENDIX II



Appendix 2: A graph of Regressional analysis of standards of PfHRP2 extracted from Adamsel.

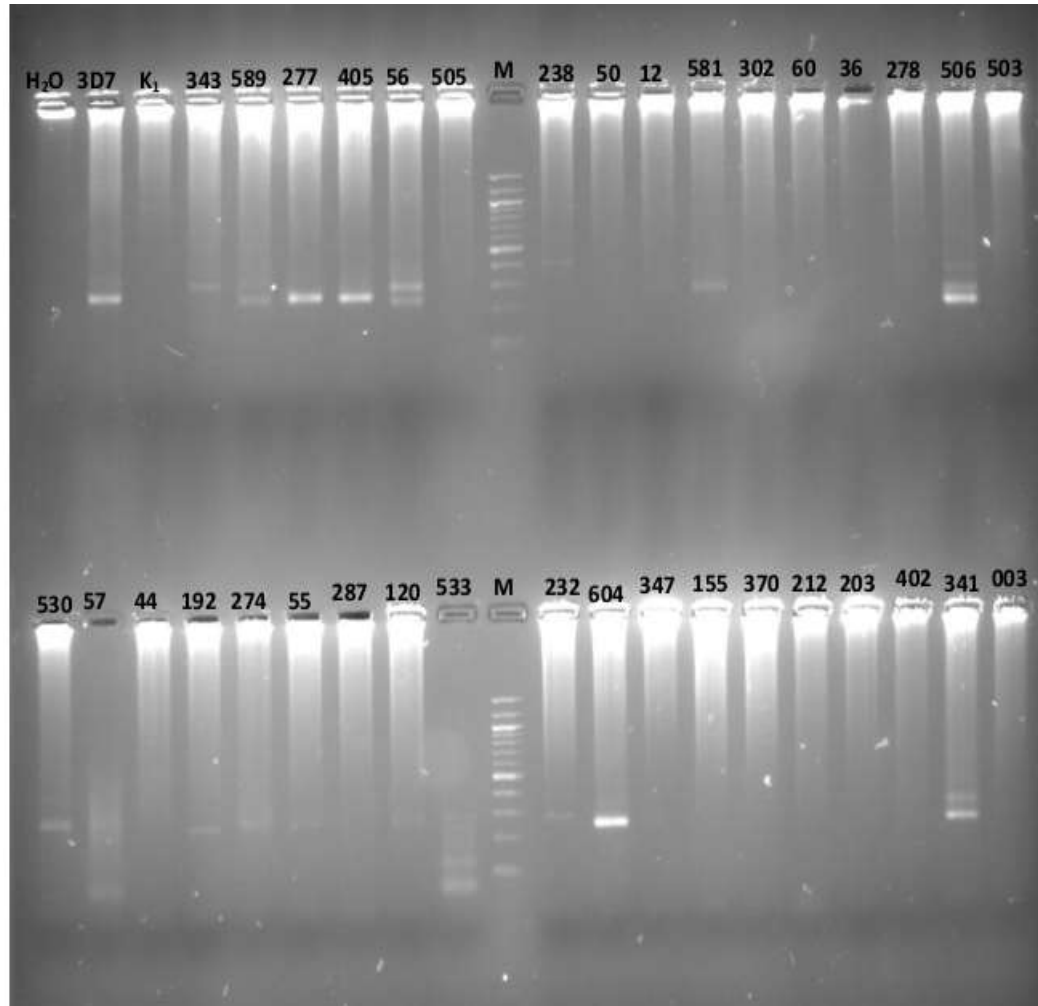
APPENDIX III

Supplementary Table 2: Regression Analysis of ELISA using Adamsel

Regression

Rsquare	0.99401
Mean Sq x 1000	0.6065
Max OD	0.39677
Scale	0.90288
Xmid	2.26092
Blank	0.059
Blank CV (%)	2.6
High cut off OD	0.397
Low Cut off OD	0.061
Calculated [Std]	123.15
N Std. incl.	12
Od = 1 + blank @	Nc

APPENDIX IV



Appendix 4 is an image showing how an *PfmSP2* gel image depicted for each gel. Each band in the image showed that the sample was positive for *PfmSP2* while wells without bands were shown be negative. Some samples had two or three bands showing there were two or three different parasite strains in each sample respectively. Numbers on top of the wells were sample IDs while M was the DNA marker. K1 and 3D7 were positive controls while H₂O was negative control.

APPENDIX V

Supplementary Table 4: List of all primers used for the study.

PCR Reaction	Nest and Product size	Primer	Neucleotide sequence	Annealing Temperature
<i>18s rRNA</i>	Nest 1	rPlu6 (F)	5'- TTAAAATTGTTGCAGTTAAAAC G-3'	55 °C
		rPlu5 (R)	5'- CTTGTTGTTGCCGTTAAACTTC- 3'	
	Nest 2 205 bp	rFAL1 (F)	5'- TTAAACTGGTTTGGGAAAACCA AATATATT-3'	58 °C
		rFAL2 (R)	5'- ACACAATGAACTCAATCATGAC TACCCGTC-3'	
<i>pfmsp2</i> Primers are semi-nested family-specific (Falk et al., 2006)	Nest 1 280 bp	M2-OF	5'- TGAAGGTAATTTAAAACATTGTC TATTATA-3'	54 °C
		M2-OR	5'- CTTTGTTACCATCGGTACATTCT T-3'	
	Nest 2 380 bp	S1fw	5'- GCT TAT AAT ATG AGT ATA AGG AGA A -3'	55 °C
		M5rev	5' - GCA TTG CCA GAA CTT GAA-3'	
		N5rev	5' - CTG AAG AGG TAC TGG TAG A-3'	
<i>Glurp</i>	Primary	GF3	5' -	

(Semi-nested with GF4 common to both)	600 bp		ACATGCAAGTGTGATCCTGAA – 3’	54 °C
		GF4	5’– TGTAGGTACCACGGGTTCTTGT GG – 3’	
	Secondary1 2400 bp	GNF	5’– TGTTCACTGAACAATTAGAT TTAGATCA–3’	
	Nest 1	2.1	5’-	
<i>pflhrp2</i> (PF3D7_08 31800)	245-308 bp		GGTTTCCTTCTCAAAAAATAAA G-3’	54.8 °C
		2.2	5’- CGAAACTCAAGCACATGTAGA- 3’	
	Nest 2 245-700 bp	2.3	5’- GTATTATCCGCTGCCGTTTTTGC C-3’	60 °C
		2.4	5’- TTCCGCATTTAATAATAACTTG TGTAG-3’	
<i>pflhrp1-2</i> (PF3D7_08 31800)	Primary 228-809 bp	2e12F	5’- CAAAAGGACTTAATTTAAATAA GAG-3’	57 °C
		2e12R	5’- TGCTACGCCATTAATTTATT- 3’	
Upstream (PF3D7_08 31700)	Primary 201-220 bp	<i>MAL7P1</i> .228F	5’- AGACAAGCTACCAAAGATGCA GGTG-3’	60 °C
		<i>MAL7P1</i> .228R	5’- TAAATGTGTATCTCCTGAGGTA	

		GC-3'		
Downstream	Primary	<i>MAL7P1</i>	5'-	
	301-405	.230F	GATATCATTAGAAAACAAGAG	58 °C
(PF3D7_08	bp		CTTAG-3'	
31900)		<i>MAL7P1</i>	5'-	
		.230R	TATCCAATCCTTCCTTTGCAAC	
			ACC-3'	
<i>pfhrp3</i> -exon	Nest 1	3.1	5'-	
	311 bp		GGTTTCCTTCTCAAAAAATAAA	54.8 °C
2			A-3'	
(PF3D7_13		3.2	5'- TAAGTCAAGCACATGCAGG -	
72200)			3'	
	Nest 2	3.3	5'-	
	201 bp		AATGCAAAAGGACTTAATTC-3'	62 °C
		3.4	5'- ACTACGCATCACTTACACCA	
			-3'	

Oligonucleotide primers and nuclease free water for the nested PCR assay were obtained from Inqaba Biotec West Africa Limited (Ghana). These primers were designed based on the *Plasmodium* small subunit ribosomal RNA (*18s rRNA*) gene.

APPENDIX VI


ETHICAL CLEARANCE AND CONSENT FORMS

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1978A Constituent of the College of Health Sciences

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Legon, Accra
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E-mail: info@noguchi.ug.edu.gh
Telex No: 2530 UGIL GH
My Ref. No: NH/13
Your Ref. No:

INSTITUTIONAL REVIEW BOARD



(NMIMR – IRB, Chair)

2nd May 2016

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 084/12-13 *reval. 2016* **ICRG 0000908**

On 2nd May, 2016, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting conducted continuing review and renewed your protocol titled:

TITLE OF PROTOCOL : **Evaluation of polymorphisms in *pfhrp-2* and *pfhrp-3* genes and subsequent efficacy of PfHRP 2 - based malaria Rapid Diagnostic Test (RDT) in Ghana**

PRINCIPAL INVESTIGATOR : **Dr. Linda E. Amosh**

CO-P. INVESTIGATORS : **Samuel Badu Nyarko**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally/ fourteen days in writing.

This certificate is valid till 1st May, 2017. You are to submit annual reports for continuing review.

Signature of Chair:
Mrs. Chris Dadzie
(NMIMR – IRB, Chair)

Volunteer Agreement Form

Protocol (1) 2014-15

version 1.0

14/8/2014

Parental Consent for children with Malaria

Title: *Plasmodium falciparum* gametocytogenesis

Principal Investigators: Dr Linda Eva Amoah

Address: Department of Immunology, NMIMR, Box LG581, Legon

General Information about Research

Malaria is common in Ghana. When you have malaria, it is important that we treat you and kill all the malaria parasites in your blood. It is also important to make sure that other people do not get sick with malaria. In order to do this, we want to study how the malaria parasites grow and move from one person to the other. We will take a small drop of blood (100 μ L) from your child's finger to see if you have malaria parasites by microscopy. If you do, we will ask for an additional half a teaspoon (2.5 ml) of venous blood for children under 6 years or one teaspoon (5 ml) of venous blood for children over 6 years will be collected for further analysis. We would also like to know if your child is better after a week or still have some malaria parasites. So we will request you come back for free screening for malaria in 7 days. In addition to the routine laboratory tests that you do when you go to the hospital with malaria, we will take some of your blood back to the NMIMR for more tests including sickling, blood typing and measuring your immune response against malaria parasites

Possible Risks and Discomforts

Your child may experience mild discomfort and bruising is possible at the site where the fingerpick and venous blood samples will be obtained. This will however resolve within an hour or two.

Possible Benefits

There are no direct benefits to your child. However, your participation may help us develop better malaria treatment.

Confidentiality

All information gathered would be treated in strict confidentiality. We will protect information about your child taking part in this research to the best of our ability. The child will not be named in any reports. However, Dr. Linda Eva Amoah may sometimes look at her research records. If you have any questions, please feel free to ask the clinician in charge.

Compensation

Your child will not be paid for participation in this study but your transportation to the follow up visit will be refunded.

Voluntary Participation and Right to Leave the Research

We would like to stress that this study is strictly voluntary. Should your child decide not to participate; it will have no consequences for him/her. Should your child, at any point during the study, decide that he/she does not wish to participate any further, participation will be terminated immediately. Any such decision will be respected without any further discussion. The decision to end participation will not affect the health care your child would normally receive.

Contacts for Additional Information

If you ever have any questions about the research study or study-related problems, you may contact Dr. Linda Amoah of the Noguchi Memorial Institute for Medical Research (0279271632) at any time.

Your rights as a Participant

This research has been reviewed and approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB). If you have any questions about your child's rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.mimcom.org.

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title (*Plasmodium falciparum gametocytogenesis*) has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to allow my child to participate as a volunteer.

Date
guardian

Signature or mark of parent or
guardian

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

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

Date

Signature of Witness

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

Date

Signature of Person Who Obtained Consent

Parental Consent for children with Malaria

Title: *Plasmodium falciparum* gametocytogenesis

Principal Investigators: Dr Linda Eva Amoah

Address: Department of Immunology, NMIMR, Box LG581, Legon

General Information about Research

Malaria is common in Ghana. When you have malaria, it is important that we treat you and kill all the malaria parasites in your blood. It is also important to make sure that other people do not get sick with malaria. Sometimes people have a lot of malaria parasites but are not sick, these people help spread malaria without knowing. We want to understand how people who are not sick help spread malaria. We will take two drops (200µl) of blood from your finger every other week to see if you have malaria parasites by microscopy. If you do, we will ask for an additional quarter teaspoon (1 ml) of venous blood for laboratory analysis. We will take your blood back to the NMIMR for testing, including sickling, blood typing and measuring your immune response against malaria parasites

Possible Risks and Discomforts

You may experience mild discomfort and bruising is possible at the site where the fingerpick and venous blood samples will be obtained. This will however resolve within an hour or two.

Possible Benefits

There are no direct benefits to you. However, your participation may help us develop better malaria treatment.

Confidentiality

All information gathered would be treated in strict confidentiality. We will protect information about you taking part in this research to the best of our ability. You will not be named in any reports. However, Dr. Linda Eva Amoah may sometimes look at her research records. If you have any questions, please feel free to ask the clinician in charge.

Compensation

You will not be paid for participation in this study.

Voluntary Participation and Right to Leave the Research

We would like to stress that this study is strictly voluntary. Should you decide not to participate; it will have no consequences for you. Should you, at any point during the study, decide that you does not wish to participate any further, participation will be terminated immediately. Any such decision will be respected without any further discussion. The decision to end participation will not affect the health care you would normally receive.

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VOLUNTEER AGREEMENT

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Date

Signature or mark of volunteer

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

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

Date

Signature of Witness

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

Date

Signature of Person Who Obtained Consent