EVALUATION OF THE PERFORMANCE OF HISTIDINE-RICH PROTEIN-2 BASED RAPID DIAGNOSTIC TESTSAND ALLELIC DIVERSITY OF *PLASMODIUM FALCIPARUM* GENES IN IBADAN, SOUTHWESTERN NIGERIA

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MATRIC No.: 159614

JULY, 2019.

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ABSTRACT

Misdiagnosis of malaria exposes patients to drug pressure which may contribute to the emergence of anti-malarial drug resistance. The WHO recommends the use of malaria Rapid Diagnostic Tests (mRDTs) to improve malaria diagnosis in resource limited settings. However, deletion of *Plasmodium falciparum* Histidine Rich Protein-2 and 3 (*Pf*hrp-2/3) gene contribute to the proportion of false negative mRDT results. Also, information on the allelic diversity of *Plasmodium falciparum* genes are essential in understanding the parasite dynamics and control mechanisms. In this study the PfHRP-2-based mRDT sensitivity, *Pf*hrp-2/3 gene deletion and the allelic diversity of merozoite surface proteins 1 and 2 (*msp1*/*msp2*) and glutamate-rich protein (*glurp*) genes in Ibadan, southwest Nigeria were evaluated.

Ethical approval was provided by the UI/UCH ethics committee (UI/EC/12/0279) and a written informed consent obtained from parents/guardians before patients enrolment. Finger-prick blood samples were collected from 511 febrile children age 3–59 months in a Primary Healthcare Center, Idi-Ayunre and St. Mary's Catholic Hospital Eleta, Ibadan for mRDT screening, thick blood smears for microscopy and Dried Blood Spot (DBS) on filter paper (Whatman 3mm) for molecular genotyping. The mRDT positive children were treated with artesunate-amodiaquine at standard dosage. Giemsa-stained blood films were examined microscopically and genomic DNA was extracted from DBS using commercial kit. Nested PCR was performed using specific primers to amplify the 18SrRNA, exon-2 regions of *Pf*hrp-2/3, *msp1*, *msp2* and *glurp* genes. The sensitivity, specificity, positive/negative predictive values (PPV/NPV), accuracy and kappa's value of mRDT was estimated using microscopy as the gold standard. The DNA sequence of *Pf*hrp-2/3 genes were determined by SANGER method and the sequencesobtained were analyzed using BioEdit software. Data were analyzed using descriptive statistics and Chi-square at $\alpha_{0.05}$.

Prevalence of malariausing mRDT, microscopy and PCR was 59.6%, 43.5% and 51.3 % respectively ($\rho < 0.001$). Based on PCR, *P. falciparum* accounted for 91.6% (228/249; alone/or in combination) while 23.3% (57/249) occurred as *P. malariae*, *P. ovale* or mixed infection with *P. falciparum*.*P. vivax* was not detected. The sensitivity of mRDT against microscopy was 95.2%, specificity 67%, accuracy 79%, PPV 68%, NPV 94.9% and kappa's statistics was 0.58. Similarly, PCR performance against microscopy was; sensitivity 84%, specificity 67%, accuracy 76%, PPV 73%, NPV 80% and kappa's statistics 0.51. Thirty one samples (12.4%) were RDT negative (false negative) but PCR positive out of which 8 (25.8%)and 4 (12.9%) had *Pf*hrp-2 and 3 gene deletion, respectively. The sequenced*Pf*hr*p*-2/3 PCR products were successfully translated to amino acid. The *Pf*hrp-*2*/*3* amino acid repeat

sequences were highly diverse. Extensive allelic diversity of *P. falciparummsp1,msp2* and *glurp* genes were also observed. The RO33 and 3D7 allelic familiesof *msp1* and *msp2*were the most predominant. The allelic frequency for *msp1*, *msp2* and *glurp* was 80.1%, 68.5% and 50.9%, respectively.

The presence of parasites lacking *Plasmodium falciparum* histidine rich protein - 2 and 3 genes and extensive allelic diversity are contributory factors for the false negative results associated with malaria Rapid Diagnostic Tests and high transmission of malaria respectively in Ibadan.

Keywords: Malaria rapid diagnostic Tests, *Pf*hrp-2/3 Deletion, *Plasmodium falciparum* genetic diversity.

Word Count: 498

DEDICATION

To my lovely mother& late father for laying a solid foundation in my life

My wife Erekposeigha

My priceless daughters and son; Catherine, Mary-Ann, Elizabeth and Roland Jr.

And finally,

The God sent super mentor Prof. Catherine O. Falade.

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MAY GOD ALMIGHTY BLESS YOU ALL!!!

CERTIFICATION

We certify that this work was carried out by Mr. **R.I. Funwei** in the Department of Pharmacology and Therapeutics, University of Ibadan

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TABLE OF CONTENT

CHAPTER TWO

CHAPTER THREE

CHAPTER FIVE

CHAPTER SIX

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

List of Abbreviations

µL	Micro-Litre
3SR	Self-Sustained Sequence Replication
ACT	Artemisinin Combination Therapy
AMA	Apical Membrane Antigen
AMV	Avian Myeloblastosis Virus
Bst	Bacillus stearothermophilus
CSP	Circumsporozoite Surface Proteins
DBS	Dried Blood Spots
DdRp	DNA dependent RNA polymerase
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphate
dTMP	deoxy-Thymidine Monophosphate
EBA	Erythrocyte Binding Antigen
FMoH	Federal Ministry of Health
FN	False Negative
FP	False Positive
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
GLURP	Glutamate Rich Protein
GMMs	Genetically Modified Mosquitoes
GPI	Glycophosphatidylinositol
IAMRAT	Institute for Advanced Medical Research and Training

IFA	Immunofluorescence Assay
IPTp	Intermittent Preventive Treatment of Malaria in Pregnancy
IRS	Indoor Residual Spraying
K13	Kelch 13
kDa	Kilo Dalton
LAMP	Loop Mediated Isothermal Amplification
LAUTECH	Ladoke Akintola University of Technology
LDR-FM	Ligase Detection Reaction Fluorescent Microsphere
LLITNs	Long Lasting Insecticide Treated Nets
LOD	Limit of Detection
mAbs	Monoclonal Antibodies
MDR	Multi-Drug Resistance
MOI	Multiplicity of Infection
MSA	Merozoite Surface Antigen
MSP	Merozoite Surface Protein
NASBA	Nucleic Acid Sequence-Based Amplification
NAT	Nucleic acid amplification technique
NMCP	National Malaria Control Program
NPV	Negative Predictive Value
PCR	Polymerase Chain Reaction
*Pf*AMA	*Plasmodium falciparum* Apical Membrane Antigen
Pfcrt	*Plasmodium falciparum* Chloroquine Resistance Transporter
Pfdhfr	*Plasmodium falciparum* Dihydrofolate Reductase

Pfdhps	*Plasmodium falciparum* Dihydropteroate Synthtase
*Pf*HRP	*Plasmodium falciparum* Histidine Rich Protein
Pfmdr	*Plasmodium falciparum* Multi-Drug Resistance
PfRH	*Plasmodium falciparum* Reticulocyte Binding-like Homologous
pLDH	Parasite Lactate Dehydrogenase
PPV	Positive Predictive Value
QBC	Quantitative Buffy Coat
qPCR	Quantitative Polymerase Chain Reaction
RBCs	Red Blood Cells
RDTs	Rapid Diagnostic Tests
RFLP	Restriction Length Fragment Polymorphism
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RT-PCR	Real-Time Polymerase Chain Reaction
SERCA	Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase
SMC	Seasonal Malaria Chemoprevention
SNP	Single Nucleotide Polymorphism
SOPs	Standard Operating Procedures
SP	Sulfadoxine-pyrimethamine
ssrRNA	Small sub-unit ribosomal Ribonucleic Acid
STRs	Short Tandem Repeats
T3	Test Treat and Track
TAS	Transcription-based Amplification System

TMD	Transmembrane Domains
TN	True Negative
TP	True Positive
TSR	Thrombospondin-like type I Repeat
UCH	University College Hospital
UV	Ultraviolet
WBCs	White Blood Cells
WHO	World Health Organization

CHAPTER ONE

1.0 Introduction

1.1 Background to the study

Human malaria infection is caused by a unicellular parasite of the genus *Plasmodium* which is transmitted by the female anopheles mosquitoe. About 90% of malaria prevalence occurs insub-Saharan Africa(WHO, 2016a). There are five human *Plasmodia species* that are pathogenic to human, these include *Plasmodium falciparum*, *P. vivax, P. ovale*, *P.malariae* and *P.knowlesi*. *Plasmodium vivax* and *Plasmodium ovale* cause relasping malaria through hypnozoite formation which mayremain dormant in the hepatocyte of the human host for several months to years (de Araujo *et al.,* 2012; Campo *et al.,* 2015; Groger *et al.,* 2017). Numerous reports of*P. knowlesi*infection has occured in man but from the island of Borneo in Southeast Asia (Singh & Daneshvar, 2013). Recently, an outbreak of human malaria caused by *P. simium,* a sixth specie was reported in Brazil, South America (de Alvarenga *et al.,* 2018). Among the established five malaria species that infect man, *P. falciparum* infections alone or as co-infection account for over 90% of malaria infections in Nigeria and Sub Saharan Africa (Dawaki *et al.,* 2016; Snow *et al.,* 2017).

Between 2000 to 2015, a subtantial reduction (41%) in malaria case incidence was recorded with 62% decline in malaria motality rates globally (WHO, 2018). These achievements are atrributable to the interventions and increased malaria control efforts in the preceeding decade. Despite the remarkable reduction in the global malaria burden, an estimated 219 million documented malaria incidence occured globally in 2017, causing about 435,000 deaths among under five yeras old in sub-Saharan Africa (WHO, 2018).

In Nigeria, malaria is transmitted all year round putting about 97%of the total populationat risk. It is a potential life-thretening disease, causing 60% of outpatient attendance and 30% of hospitalization (PMI, 2015). The estimated malaria mortalityin under five years old children in Nigeria is about 225,000 annually, while malaria is estimated to be responsible for about 11% maternal mortality and 10% low birth weights (FMoH, 2014). The ecolozical zones that determines malaria intensity and seasonality of transmission is defined by the magroove swamp, rain forest, Guinea, Sudan and Sahel Savanah. Malaria transmission rates varies across these ecological zones with a seasonal decrease

throughout the year in the south and about three months or less in the northern part of Nigeria(FMoH, 2014).

The burden of malaria can be substantially reduced with accurate diagnosis and appropriate management with efficacious antimalarial drugs. Parasite based diagnosis of malaria is essential for epidemiological mapping andsurveillance in guilding drug efficacy, vaccine trials and other interventional programmes. The basis for an accurate diagnosis of malaria requires adefinitive detection of the parasite, differentiation of the infecting parasite species, quantification of parasitaemia and monitoring of drug efficacy outcomes of antimalarials (Baraka *et al.,* 2018; Mat Ariffin *et al.,* 2017). Parasite-based malaria diagnosis can be achieved routinely with malaria rapid diagnostic tests (RDTs) and microscopy in both field and clinical settings while polymerase chain reaction (PCR), is more applicable for reseach purposes and in reference laboratories due to its complex opperational methodology (Johnston *et al.,* 2006).

Presumptive malaria treatment accounts for a very high proportion of treated febrile illnesses suspected to be malaria infection in sub-Saharan Africa(Chandramohan *et al*., 2002; D'Acremont *et al*., 2009), resulting in over prescription and use of antimalarial drugs that may lead to poor disease monitoring and emergence of parasite selection pressure (Nzila & Mwai, 2010). Presumptive malaria diagnosis is non-specific because of the similarities of the presenting signs and symptoms with other common febrile illnesses like urinary tract infection, pneumonia, influenza, dengue fever, common cold and upper respiratory tract infections (WHO, 2010). Therefore, parasite-based diagnosis will facilitate appropriate and prompt malaria treatment, reduce the rate of drug misuse and minimizes the risk of parasite selection pressure to antimalarial drugs(Gamboa *et al.,* 2010).

The World Health Organization has recommended that malaria treatment should be based on parasitogical confirmation of asexual parasite in all suspected malaria cases prior to medication (WHO 2010). Diagnosis by microscopy or the use of malaria rapid diagnostic tests (RDTs) are the reccommended options but malaria RDT is moreviable in achieving this policy change by targeting an antigens expressed by *Plasmodium* against monoclonal antibodies (mAbs).

Giemsa stained thick and thin blood filmsusing microscopy is the global reference standard for routine diagnosis of malaria (Bharti *et al.,* 2007; Tangpukdee *et al.,* 2009). It is affordable, sensitive and specific in the hands of expert microscopists with effective

quantification of parasitaemia in infected individuals (Aiyenigba *et al.,* 2017; Prescott *et al.,* 2012). However, there are a number of challenges with the use of microscopy which include lack of basic infrastructure, limited trained laboratory technicians (microscopists) and low sensitivity at low parasite density (Batwala *et al.,* 2011).

The introduction of RDTs has reduced presumptive malaria diagnosis and improved appropriate malaria treatment in resource limited settings. Malaria RDTs are antigen-antibody based lateral-flow immuno-chromatographic assays based on the binding affinity of specificantigens with antibodies. Targeted *Plasmodium* antigens include*Plasmodium falciparum* histidine rich protein-2(*Pf*HRP-2), *Plasmodium*lactate dehydrogenase (pLDH) and *Plasmodium*aldolase(Moody, 2002). While *Pf*HRP-2 based RDTs can only detect*P. falciparum,* pLDH and adolase RDTs can detect non-*falciparum* malaria (pan specific) (Wanja *et al.,* 2016).

Malaria RDTs has numerous advantages regardless of the setting. Malaria RDT is not laborious, electricity is not required, needs minimal operator training, test is completed within 20 minutes and results are easily interpreted with high reproducibility (Wongsrichanalai *et al.,* 2007). However, RDT performanceis limited by the product quality, storage pattern and condition, and low parasite density which may reduce RDT sensitivity (Bell *et al.,* 2006; Chiodini *et al.,* 2007).

The occurrence of RDT false positive and false negative RDT results pose major concern for RDT applicability (Kozycki *et al.,* 2017; Lee *et al.,* 2014). False positive results are mainly caused by persistence antigenaemia (Iqbal *et al.,* 2004; Mayxay *et al.,* 2001). HRP-2 antigen is reported to persist and circulate of previously infected individuals for at least two weeks and sometimes over eight weeks after asexual parasites have been cleared, leading to a false positive results (Gatton *et al.,* 2015; Tjitra *et al.,* 2001). On the other hand, false negative RDT may occur at low parasitaemia (less than 200 parasites/μL), non *falciparum,*deletion of *Pf*hrp-2 gene and/or nucleotide sequence repeats rearragement resulting from genetic polymorphism in the parasite population (Baker *et al*., 2005; Gamboa *et al*., 2010; Koita *et al*., 2012; Kumar *etal*., 2014).

Although, malaria RDTs that are specific to *P. falciparum* target *Pf*HRP-2 antigen,a high homology exists between *Pf*hrp-2 and *Pf*hrp-3 genes. This homology in amino acid sequence implies that the two genes may have evolved from a common ancestral gene or filial generation(Wellems & Howard, 1986). Some *Pf*HRP-2 based RDTs have been reported to cross-react with *Pf*HRP-3 antigens because of the amino acid sequence

similarities between these two proteins (Lee *et al.,* 2006a). Parasites lacking *Pf*hrp-2 and *Pf*hrpP-3 with active malaria infection in clinical isolates was first reported in Peru of the Amazonian region (Gamboa *et al.,* 2010) and subsequently in Colombia (Murillo Solano *et al.,* 2015), Honduras (Abdallah *et al.,* 2015) with varying proportion of deletion (4% – 41%). The presence of*Pf*hrp-2 deletion in malaria parasites in Peru was correlated with the poor *Pf*HRP-2-based RDTs sensitivity (Maltha *et al.,* 2012).

Apart from the Amazon region, a low prevalence of *Pf*hrp-2 gene deletion has been reported in India (Bharti *et al.,* 2016); along the China–Myanmar border (Li *et al.,* 2015), Africa, including Mali (Koita *et al.,* 2012), Senegal (Wurtz *et al.,* 2013), and DR Congo (Parr *et al.,* 2016). Among the reported *Pf*hrp-2/*Pf*hrp-3gene deletions in the African sub region, Eritrea and Ghana accounted for the highest prevalence (70% - 80% and 22% – 41%) respectively(Amoah *et al.,* 2016; Berhane *et al.,* 2018).

The Polymerase chain reaction (PCR) malaria diagnostic technique is a more sensitive, specific and accurate nucleic acid based method for the detection and differentiation of submicroscopic and microscopic malaria infections (Golassa *et al.,* 2015; Wong *et al.,* 2012). The small sub-unit ribosomal RNA (ssrRNA also called 18S-rRNA) gene is the principal target gene for PCR amplification. A nested PCR that targets this multi-copygene is considered standard for PCR-based parasite detection because it is highly conserved with high throughput in sensitivity and specificity (Golassa *et al*., 2015; Rougemont *et al*., 2004). The sensitivity of nucleic acid test based on using the 18SrRNA gene ranges between 1– 5 parasites/µL while microscopy and RDTs limit of detection is about 50 – 200 parasites/ µL (Echeverry *et al.,* 2016; Tangpukdee *et al.,* 2009). Despite the robustness in PCR's high diagnostic throughput, it has a complex operational methodology, high cost of reagents needing highly trained technicians (Fakruddin *et al.,* 2013a). Thus, the PCR method is usually deployed for follow up therapeutic efficacy studies to discriminate recrudescence from new infections through allelic genotyping of the infecting parasite strains andto identify drug resistance biomarkers. PCR is also useful in screening of mixed infections and the characterization of genetic diversity and polymorphism in parasite population in reference laboratories and in research settings (Valones *et al.,* 2009).

Furthermore, the emergence of parasite resistance to current antimalarial drugs (ACTs) in Southeast Asia and border countries suggests that a concerted malaria containment and control efforts needs to be instituted plus the requirement to dissect and evaluate the

genetic structure of the parasite population across geographic locations that are malaria endemic(Ashley *et al.,* 2014; Talundzic *et al.,* 2015). The continuous scalling-up of malaria programmes may result in parasite selection pressure and parasite evolution that may further compromise the efficacy of currently adopted techniques to diagnose and treat malaria. It is therefore, necessary to put in place, a continuous surveillance of biomarkers associated with polymorphism in the parasite population across geographic locations that are malaria endemic (Antony & Parija, 2016; Ashley *et al*., 2014). *Plasmodium falciparum* genes are associated with high polymorphisms, which have been used as a tool in assessing the parasite population dynamics (Färnert, 2008). The genetic composition and variability of *P. falciparum* is related to the clinical and pathological properties of the parasite such as cyto-adherance, susceptibility or resistance to drugs, parasite virulence and Immune evasion (Snounou *et al*., 1999; Sondo *et al*., 2019). Routine monitoring and evaluation of the first line anti-malaria drugs and the parasite sub-population with molecular techniques have been recommended as part of efforts in monitoring drug resistance to scale-up malaria control strategies (Shayo *et al.,* 2015).

Analyzing the genetic diversity of biomarkers is crucial to mapping out the epidemiology of malaria and its association to drug resistance by parasite strains (Peek *et al.,* 2005). This has limited the pace for developing new antimalarial drug and effective vaccine production in the quest to eradicate malaria at various time points globally (Ghansah *et al.,* 2014). The membrane-bound *Plasmodium falciparum* merozoite surface proteins (MSP1, MSP2*)* and glutamate-rich protein (GLURP*)* are molecular biomarkers with significant genetic variability that is effective in eveluating the multiplicity of infection (MOI) in variousstrains of the parasite, and to differentiate recrudescence from re-infecting parasites via allelic genotyping (Maestre *et al.*; Mwingira *et al.,* 2011). The MSP1, MSP2 and GLURP are parasite proteins that are considred for vaccine development because of the diverse role they play in the parasite's life cycle(Barry *et al.,* 2009; Duru & Thomas, 2014).

Themerozoite surface proteins (MSP1 & MSP2) are adhesive membrane-bound proteins attached to infected erythrocytes and are useful during invasion of the erythrocyte(Beeson *et al.,* 2016). The MSP1 is a major antimalarial vaccine target because antibodies produced during erythrocyte invasion triggers the immune system, resulting in the inhibition of parasite growth (Conway *et al.,* 2000; Woehlbier *et al.,* 2006). The *msp1*gene is associated with 17 blocks, comprising seven repetitive blocks that are interspersed with five conserved and five semi-conserved blocks (Miller *et al.,* 1993). It is extensively

polymorphic with genetic variations particularly in block 2 region of *msp1*, comprising three (K1, MAD20 and RO33) allelic families(Beeson *et al.,* 2016; Kolawole *et al.,* 2016). The *msp*2 gene is a dimorphic gene with two (3D7 and FC27) allelic families, both having N and C-terminals, that are specific to a particular strain and variable regions (Congpuong *et al*., 2014a; Niang *et al*., 2017). It functions during parasite invasion and remain adhesive to infected erythrocytes (RBCs) throughout the period of invasion and can only be degraded when the invasion process is completed (Boyle *et al.,* 2014).

On the other hand, the *glurp* gene comprises a conserved R0 region, a repetitive central R1 region and a C-terminal immuno-dominant repetitive R2 region (Theisen *et al.,* 1998). The B-cells present in the R2 region can stimulate antibodies that are capable of inhibiting parasite growth (Theisen *et al.,* 1995). The *msp*1 block 2,*msp*2 block 3 regions, and *glurp* R2 region are genetically characterized to elucidate the antigenic structure of the parasite population and allelic frequency distribution as a tool for molecular surveillance for monitoring of multiple parasite strains(Congpuong *et al*., 2014a; Mwingira *et al*., 2011; Ojurongbe *et al*., 2011).

1.2 Study rationale

Over 90% of malaria in Nigeria is caused by *Plasmodium falciparum*(Ademowo *et al.,* 1995; Dawaki *et al.,* 2016). The use of *Pf*HRP-2 based RDTs for *P. falciparum*detection across health facilities are recommended for programatic deployment by the National Malaria Elemination Program (NMEP) in Nigeria. However, *Pf*hrp-2 and/or *Pf*hrp-3 gene deletion and nucleotide variabilityof these genes may limit RDT applicability in malaria diagnosis *(*Berhane *et al.,* 2018*;* Gamboa *et al*., 2010; Kumar *et al*., 2014). If RDT fails to detect parasites lacking *Pf*hrp-2 gene as the infection will not be detected and therefore not treated, it may lead to progression of disease severity and possibly death. A previouly conducted multi-country study which investigated *P. falciparum* isolatesacross different continents, including Africa (Nigeria), reported high polymophism in the*Pf*hrp-2 and *Pf*hrp-3 nucleotide sequences(Baker *et al*., 2010). However, the studyhas a limited information on the democraphic details of the study participants, location(s)where the isolates were collected anddid not report*Pf*hrp-2 and/or*Pf*hrp-3 gene deletion from Nigeria. *Pf*hrp-2 and/or *Pf*hrp-3 genedeletion and nucleotide variability may thus pose a major challenge to malaria intervention programmes in Nigeria.It is therefore important to evaluate *Pf*HRP-2 based RDTs with false negative results for *Pf*hrp-2 and *Pf*hrp-3 gene

deletion and the nucleotide variability from circulating parasites in Ibadan, southwest Nigeria.

In addition, the merozoites surface proteins and glutamate-rich protein (*msp*1, *msp2,* and *glurp)* are polymorphic biomarkers widely utilized to elucidate parasite population dynamics. It is imperative to evaluate these biomarkers for continuous molecular surveillance to elucidate the genetic structure of the parasite population, pathogenesis of the disease, pattern of transmission and mechanisms associated with the emergence of drug resistance. Studies that can provide baseline data for these genes from different geographical regions are essential (Jallow *et al.,* 2009). Thus, these antigenic biomarkers warrant a systematic characterization among parasite sub population in Nigeria.

1.3 Study aim

This study is to investigate the performance of *Pf*HRP-2 based RDTs, the nucleotide sequence variability of conserved*Pf*hrp-2/*Pf*hrp-3 genes and the population genetic diversity of *msp*1*, msp*2and *glurp* genes of *Plasmodium falciparum* in Ibadan, southwest Nigeria.

1.4 Specific objectives

1 To evaluate and compare the performance of *Pf*HRP-2 based RDT against microscopy and PCR in malaria diagnosis in febrile children from Ibadan, southwest, Nigeria.

2. To determine the prevalence of *Plasmodium species*in circulation using PCR speciation

3. To determine whether RDT false negative results are linked with the spontaneous *Pf*hrp-2/*Pf*hrp-3 gene deletion or as a result of low parasitaemia.

4. To evaluate the recurrence of parasitaemia following treatment with artesunate/amodiaquine (ASAQ) to distinguish recrudescence from new infections.

5. To characterizethe circulating parasite population genetic diversity and allelic frequency distribution of *msp*1, *msp*2 and *glurp* genes and the amino acid sequence variation within the consevered regions of*Pf*hrp-2 and *Pf*hrp-3 genes.

CHAPTER TWO

2.0 Literature review

2.1 Life cycle of malaria parasite

The *Plasmodium* parasite life cycle consists a definitive (mosquito) and an intermediate (Human) host. Whiletaking its blood meal, an infected female *Anopheles* mosquito inoculates sporozoites from its salivary gland into the blood stream of humans(Gerald *et al.,* 2011). The inoculated sporozoites quickly migrate to the liver and infect hepatocytes, reproducing asexually and afterwards develop into schizonts (Aly *et al.,* 2009). Matured liver schizonts rupture and are released as merozoites into the systemic circulation. Some *Plasmodia* species such as *P. vivax* and *P. ovale* cause relapse via the formation of a dormant stage called hypnozoites. The dormant hypnozoites can be in the their inactive form in the liver for weeks to years, after which they are released to the blood stream causing a relapse of the infection (White, 2011). The liver stage asexual replication is termed pre-erythrocytic or liver stage schizogony. Once parasites are released into the bloodstream, they invade the red blood cellsand asexually reproduce (erythrocytic schizogony) (Gerald *et al.,* 2011). While in the red blood cell, immature trophozoites (ring stage) mature to form schizonts and are released as merozoites. At this stage, differentiation of the parasite to gametocytes may occur(Guttery *et al.,* 2012). Manifestation of clinical signs and symptoms that occur during *falciparum*malaria is caused by blood stage parasites that continuously infect all stages of RBCs (Cowman & Crabb, 2006).

Two types of gametocytes are formed, the microgametocytes (male) and the macrogametocytes (female) are ingested during blood meal by the*Anopheles*from infected humans. The cascade of reproductive activities inside the mosquito gut is termed sporogony (Aly *et al.,* 2009). During gametogenesis, a zygote is formed as the microgamete penetrate and fuses with the macrogametes in the gut of the mosquito. Elongation of the zygote makes it to become a motile öökinete. At the mosquito'smid-gut, the newly formed and motileöökinete is embedded and develop to form anöocysts. The newly formed öocysts develop to become mature and are released as sporozoites, which migrate to the mosquito's salivary gland. Inoculation of the sporozoites from the salivary glandinto a new individual (new infection) ensures continuity of the parasite's life cycle (Aly *et al.,* 2009).

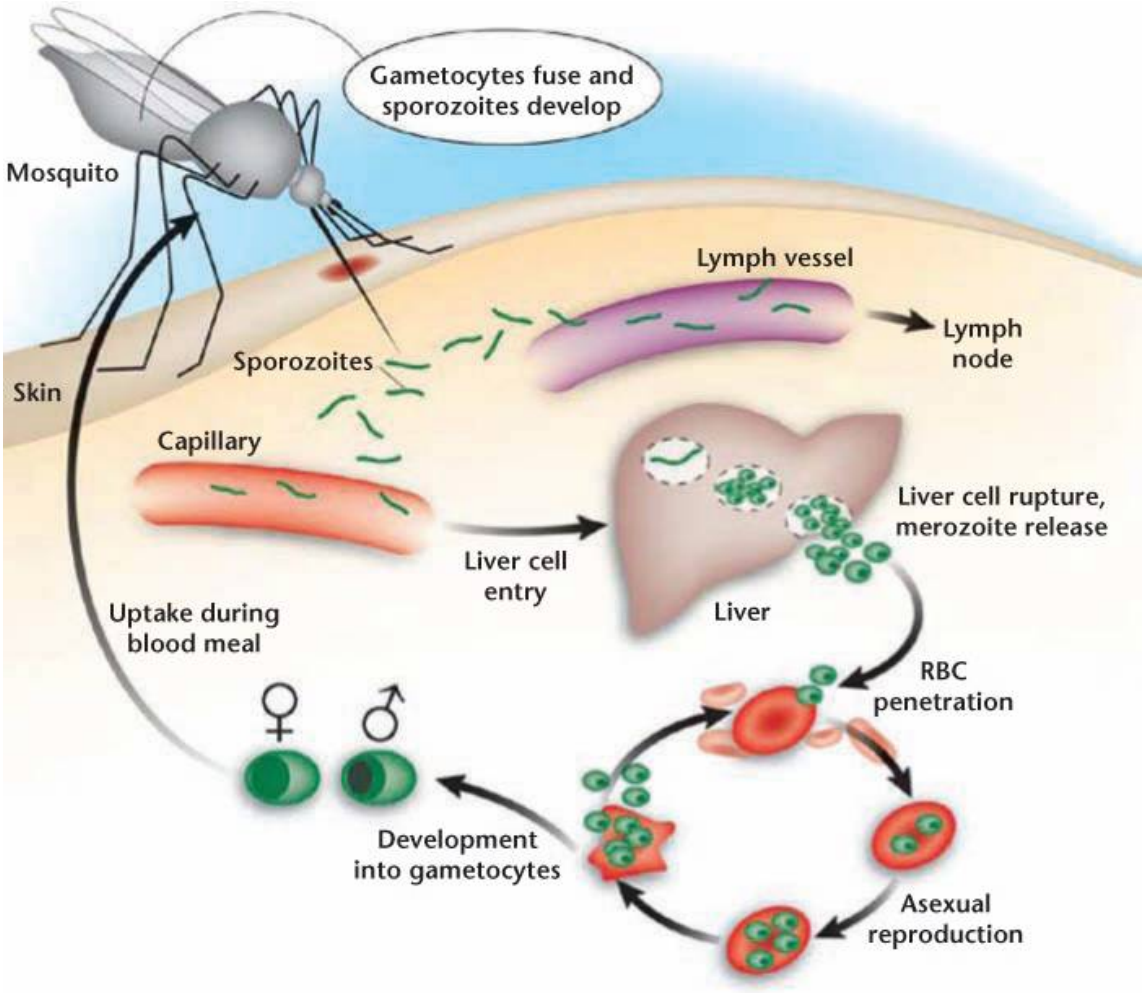


Fig. 2.1. Life cycle of malaria infection(Schantz-Dunn & Nour, 2009).

2.2 Malaria diagnosis

Accurate malaria diagnosis provides the opportunity for definitive treatment with improved care for patientsas reccommend by WHO to Test, Treat and Track (T3) all suspected malaria cases (WHO, 2012). Early diagnosis of malaria promotes effective and timely treatment which reduces the risk of disease severity and death while at the same time reducing the chances of emergence of drug resistant parasites. Laboratory confirmed malaria diagnosis is essential for all settings to guide healthcare professionals to correctly treat infected patients as misdiagnosis is seen as a significant cause of mortality. Even though, presumptivediagnosis is inaccurate and low in specificity, it is still used as the basis for antimalarial drug medication in many developing countries and in rural settings where functional laboratories and skilled manpower are limited (Choge *et al.,* 2014). Accurate malaria diagnosis willreduce irrationale administration of antimalarial drugs that may lead to drug induced adverse effects and emergence of parasite selection pressure (Khan *et al.,* 2012)

2.2.1 Malaria diagnosis by microscopy

Gustav Giemsa, a chemist and bacteriologist introduced methylene blue and eosin stains mixture in 1904 to detect malaria parasites in the blood by staining the nuclear material red and the cytoplasmic material blue at pH 7.2 (Fleischer, 2004). Microscopic detection and identification using Giemsa-stained blood smears is the gold standard for malaria diagnosis (Wongsrichanalai *et al.,* 2007). Blood smears are prepared when a finger prick drop of blood (10µL) is spotted and spread on a dry microscope slide to form thick and thin films. After allowing the smear to air dry, 10% freshly prepared Giemsa stain is dropped on the smear and placed horizontally for about 15–20 minutes. During this period of staining in the aqueous medium, red blood cells (RBCs) lyse due to osmotic pressure, detaching from the leukocytes and the parasites. The parasite from the lysed erythrocytes become more visible to be examined on the thick blood smears. Thin blood smears are fixed with alcohol before staining and are just one cell layer thick when properly prepared. The parasites are retained within the erythrocytes. Thick smears are more sensitive than thin films for diagnosis because a large volume of blood is screened while thin smears are better for speciation. The limit of detection (LOD) for thick smear is about 4–20 parasites/µL in the hand of an expert microscopists and about 50–100 parasites/µL in field conditions (Kain *et al.,* 1998; Khairnar *et al.,* 2009). However, microscopy has poor sensitivity in low parasitaemia and for asymptomatic infections, resulting in under-

diagnosis of malaria compared with molecular assays based on nucleic acid such as the polymerase chain reaction (PCR) (Wong *et al.,* 2012).

Microscopic examination of blood smear is relatively cost effective, it can differentiate and quantify parasites density during drug efficacy trials(Murphy *et al.,* 2013). However, training of expert microscopists is inadequate, reading slides is labour intensive and the occurrence of false negative at low parasite density is common (Antony *et al.,* 2013).

2.2.2 Malaria diagnosis by malaria rapid diagnostic test

Malaria rapid diagnostic tests (RDTs) are lateral-flow immuno-chromatographic assays for prompt malaria diagnosis at the point of care (Mills *et al.,* 2010). Targeted pathogens are rapidly detected with monoclonal antibodies directed against expressed antigens in the blood (Sokhna *et al.,* 2013). The test cassette is composed of a sample well, where the specimen, about 5-10µL of sputm, urine, serum plasma or whole blood is added to the sample well. This is immediately followed by the application of 2 – 4 drops of a buffer solution as specified by product manufacturers. The buffer and the sample migrate as a fluid bolus acrossthe test strip through capillary forces imbeded inthe downstream absorption surface with a conjugated pad. The conjugate contains specific antibodies coupled to a specific reporter species. The specific antibodies freely migrates with the bolus fluid because they are not immobilized. The presence of the target antigen in the sample form a conjugate complex with the reporter antibodies. The complex is absorbed bynitrocellulose membrane until they come in contact with a thin band called the test line that has an immobilized captured antibodies. The captured antibodies bind to specific epitope of the target antigen to an immuno-complex providing a visible optical output, (Lee *et al.,* 2012). The unbound reporter antibodies at the test line continue to drain along the nitrocellulose membrane until they are bound by antibodies immobilized in thecontrol line. Generally, RDTs have numerous advantage in malaria diagnosis. They are simple to perform, have a quick turnaround time and required minimal training(Hansen *et al.,* 2015).

i *Plasmodium falciparum* Histidine Rich Protein-2 antigen

The *Plasmodium falciparum* histidine rich protein-2 (*Pf*HRP-2) is a water soluble and heat-stable protein with a molecular mass of 30 kDa, it is exclusively expressed by *P. falciparum* at the asexual stage and by immature gametocytes (Tjitra *et al.,* 2001). They

are abundantly expressed by *P. falciparum*and continue to persists in the blood after the clearance of asexual parasites from previously infected individuals(Gatton *et al.,* 2015; Laurent *et al.,* 2010). The parasite biomass contributes to the protein concentration and its duration of persistence (Gatton *et al.,* 2015). The *Pf*HRP-2 is composed of 12 % aspartate, 35% histidine and 40% alanine but variation of amino acid percentages may exist depending on the infecting strain of the parasite (Lee *et al.,* 2006a). Commercially developed monoclonal antibodies (MAbs) targeting *Pf*HRP-2 based RDTs are commonly available for use (Kattenberg *et al.,* 2012). A major factor that determines RDT sensitivity and specificity is the binding affinity of the MAbs (Leow *et al.,* 2014),with the number of target epitopes expressed by the parasite, where an epitope with higher frequency may produce greater sensitivity. The frequency and universal distribution of specific target epitopes in the parasite sub populationis a key factor on the efficiency of RDT performance (Jimenez *et al.,* 2017). These targeted epitopes must be in high prevalence with copy number (Schachterle *et al.,* 2011). It is also plausible that due to the extensive polymorphism of hrp-2 nucleotide sequence, variability in the target epitopes may exist among parasites from different geographic locations, which may also determine RDT performance (Baker *et al*., 2010; Forney *et al*., 2001).

ii. *Plasmodium* Lactate Dehydrogenase (pLDH)

Plasmodium Lactate Dehydrogenase is an essential enzyme in the glycolytic pathway of *Plasmodia* species. It is produced both in the pre-erythrocytic and erythrocytic stages (Makler *et al.,* 1998; Moody, 2002; Tjitra *et al.,* 2001). The *Plasmodia* parasite and erythrocytescannot produce mitochondrial ATP via the citric acid cycle, hence, it utilizes glucose from anaerobic metabolism by making pLDH a unique and essential enzyme for the parasite for its energy requirement (Gladden, 2004). The parasite specific LDH is almost homologous with human LDH, sharing about 20% amino acid sequence similarity and a conserved catalytic residues for enzymatic activity which shares about 90% amino acid sequence similarity in all *Plasmodia species*. All parasite species shares the same epitopes, therefore pLDH-based RDTs are sensitive to all infecting species of the parasite which can be utilized in detecting non-*falciparum* infections (Heutmekers *et al.,* 2012). Pan-specific malarial RDTs probably use common epitopes inLDH against MAbs. Although,, *P. falciparum* and *P. vivax* LDH vary in their amino acid sequences and must be considered when preparing antibodies against target epitopes of specific peptides in discriminating the two species and the panel of MAbs that can differentiate all *Plasmodia* species (Feleke *et al.,* 2015). Accurate detection of all species with a pan-specific RDT

reduces false negative results with an improved sensitivity to detect non-*P. falciparum* malaria (Abba *et al.,* 2014).

Several studies have been carried out on the evaluation of multiple pLDH-based RDTs (Ashton *et al.,* 2010; Barber *et al.,* 2013). These could either bespecific to *P. falciparum* and *P. vivax*, or can be triple tests for *P.vivax*-pLDH, *Pf*pLDH and *Pf*HRP-2 or four line tests that consists of*Pf*HRP-2, pan-pLDH and *P. vivax*-pLDH and a control (Ashton *et al.,* 2010). The pLDH specific RDTs are less sensitive and perform poorly at low parasitaemia. *Plasmodium* LDH sensitivity is positively correlatedwith parasitaemia (Hendriksen *et al.,* 2011) and are less sensitive compared to *Pf*HRP-2 based RDTs (Grandesso *et al.,* 2016).

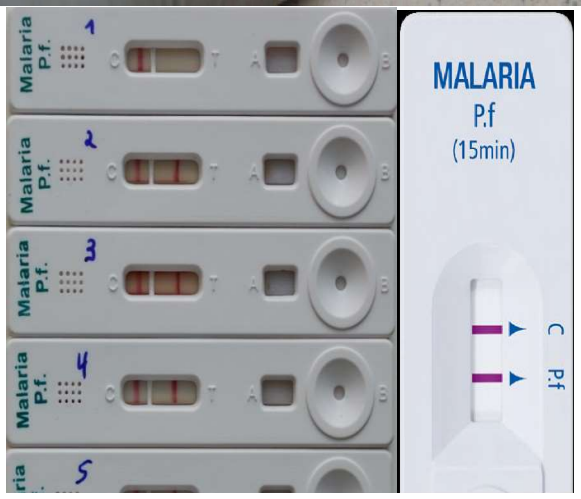
One major advantage of pLDH based RDTs is that, the target enzyme clears from blood immediately after parasite clearance.Thus pLDH based RDTs are often deployed for drug efficacy trials alongside microscopy to monitor therapeutic responses of a test drug for predicting treatment failure (Nyunt *et al.,* 2013). Also, the pLDH amino acid structure is highly conserve, it lacks antigenic variation and exhibit no prozone effect (Gillet *et al.,* 2009). However, pLDH based RDTs has decreased sensitivity at low parasitaemia and false positive result may occur due to high gametocytaemia (Murray *et al.,* 2008)

iii. *Plasmodium* Aldolase

Aldolase is an enzyme in the glycolytic pathway of the malaria parasite and in the host tissues of the parasite. It catalyzes dihydroxyacetone phosphate and glyceraldehyde-3 phosphate formation from fructose 1, 6-bisphosphate (Choi *et al.,* 2001). The aldolase expressed by *P. falciparum* parasite shares about 61–68% amino acid sequence similarity with eukaryotic aldolases (Knapp *et al.,* 1990). The aldolases of *P. vivax* and *P. falciparum* are relatively conserved with about 369 amino acid length each (Cloonan *et al.,* 2001). A single nucleotide polymorphism at position 180 of the *P. vivax* aldolase gene has been identified (Cloonan *et al.,* 2001; Lee *et al.,* 2006b). There are limited studiesinvestigating aldolase based RDTs for malaria diagnosis compared to the numerous data on *Pf*HRP-2 and pLDH-based RDTs. A combination of aldolase with*Pf*HRP-2 based malaria RDTs for *P. falciparum* and non-*P. falciparum* species diagnosis yielded poor sensitivity for the aldolase test line (Moody, 2002). The concentrations of aldolase released by the parasite into host blood is low, thus its sensitivity and specificity levels are based on parasite biomass (Barber *et al.,* 2013). Aldolase based RDTs has poor heat

stability which can be easily degraded if not properly stored at the recommended storage temperature (WHO, 2010).


C
Pf
MALARIA
P.f
ALEX


Malaria P.f.
1
2
3
4
5
C
T
A
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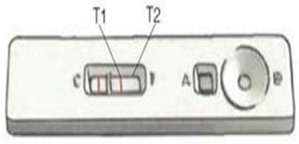
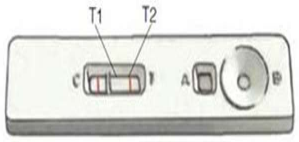
MALARIA
P.f
(15min)
C
P.f


T1
T2
C
T
A
B

Pf +


T1
T2
C
T
A
B

Non-Pf +

Fig 2.2. RDT Cassettes placed in a smooth horizontal surface. After finger prick, 5µL blood is collected with an applicator to the sample well. Four drops of the assay diluent is added into the buffer well then allowed to migrate along the nitrocellulose membrane for about 15 – 20 minutes.The appearance of ‘’C’’ (Control line) = Negative. Two bands ‘’C’’ &‘’T’’(Test line) = Positive while diskettes without “C” (control) line was considered invalid.

Source: SD-Bioline Fact Sheet 2019.(www.abbott.com/poct)

2.2.3 Molecular diagnosis of malaria

Nucleic acid amplification techniques (NATs) are the most sensitive diagnostic methods currently utilized for research purposes and in reference laboratories for confirmatory diagnosis of malaria (Proux *et al.,* 2011). NATs can detect infections that are asymptomatic and difficult to diagnose with microscopy and RDTs. The reported limit of detection (LOD) for NATs ranges from 1 – 5 parasites/μL(Perandin *et al.,* 2004). There are several molecular techniques used in detecting malaria parasites.

DNA amplification by PCR has shown a profound sensitivity for parasite detection. The specificity inherent to this method allows the unequivocal identification and distinguishing of different parasite species(Lau *et al.,*2015). PCR-based diagnostic method targets the amplification of the small subunit rRNA (18s-rRNA) gene which is a conserved house-keeping gene with multi-copy numberfor molecular diagnosis of all human *Plasmodia* species (Rougemont *et al.,* 2004). The efficiency of the assay is significantly improved when a nested PCR technique is adopted. In this methodology, two rounds of PCR amplification is conducted, utilizing the results of the primary reactionas a template for the nested reaction. Sequence specific oligonucleotide primers targeting the region or gene of interest are used to hybridize the sequences contained in the product (Wang *et al.,* 2014). PCR has the potential of amplifying a single parasite genome into billion copies of DNA that can anneal according to base pair sizes and can be distinguished routinely and reproducibly. The sensitivity of PCR amplification depends on the concentration and quantity of the initial DNA template. Nested PCR amplification has become the standard for nucleic acid based malaria diagnosis because of the multiple copy number of the 18SrRNA with high sensitivity and specificity (Echeverry *et al.,* 2016).

PCR-based malaria diagnosis are more advantageous compared to microscopy and RDT. It is nucleic acid based and is highly sensitive and specific with high-throughput and are used in screening mixed *Plasmodia* infections (Imwong *et al.,* 2014). However, running multiple PCR assays and the rigors of band size identification from different PCR products makes nested PCR to be cumbersome and time consuming. In addition, the cost of the equipment, reagents and technical expertise required to carry out PCR procedures has limited its routine and field applicability.

The development of quantitative real-time PCR (qPCR) is an advancement on the conventional nested PCR (Deepak *et al.,* 2007) as it gives an immediate output as the

reaction is in progress. A minimal amount of DNA can be used to start the reaction but the amplified end products can be accurately quantified (Imwong *et al.,* 2014). Unlike conventional PCR, there is no post processing of PCR amplicons (gel electrophoresis) which saves resourses and time. The sensitivity of qPCR is well documented in the identification and quantification of all *Plasmodia*species, including *Plasmodium knowlesi* with primers and species-specific probes targeting the 18S rRNA gene (Mangold *et al.,* 2005; Rougemont *et al.,* 2004). Despite the high costs associated with qPCR that limits its applicability in resource-poor settings, its robustness and rapid parasite quantification warrants its use as a diagnostic adjunct for parasitic infection in clinical trials (Andrews *et al.,* 2005; Chen *et al.,* 2004; Ojurongbe *et al.,* 2012).

The Loop Mediated Isothermal Amplification (LAMP) is a well-established molecular technique for pathogen detection that was first invented byNotomi(Notomi *et al.,* 2000). LAMP can rapidly amplify few copies of DNA to as many as 10^9 in a short space of time (< 1 hour) isothermally with high sensitivity and specificity (Mori *et al.,* 2006). The enzyme, Bacillus stearothermophilus (Bst) DNA polymerase is used to catalyse the LAMP reaction in an isothermal conditions (Chander *et al.,* 2014). At present, LAMP is extensively used in the amplification of DNA for detection or diagnostic purposes of all pathogens such as parasites, bacteria, fungi and viruses (Fu *et al.,* 2011). Based on results from previous results from previous reports on the sensitivity of pathogen detection, LAMP is considered one of the high molecular technique for the rapiddetection of bio-samples at the point of care (Abdul-Ghani *et al.,* 2012; Cook *et al.,* 2015; Mori &Notomi, 2009).

The Nucleic acid sequence-based amplification (NASBA) is also called the "self-sustained sequence replication" (3SR). It is a combination of primers and transcription-based amplification system (TAS) for the replication of nucleic acids in a specific single mixture (Fakruddin *et al.,* 2013b). The entire reaction is performed at a constant temperature at 41°C. Three unique enzymes are used in the NASBA reaction: The avian myeloblastosis virus (AMV) reverse transcriptase (RT), RNase H and T7 DNA dependent RNA polymerase (DdRp). The integration and utilizationof RT amplification process makes the technique appropriate for RNA and genomic DNA studies (Compton, 1991; Deiman *et al.,* 2002).

The Ligase Detection Reaction Fluorescent Microsphere (LDR-FM) assay is a multiplex reaction for identifying multiple point mutations associated with *P. falciparum* at

different sites of the target gene (Nankoberanyi *et al.,* 2014), with a more cost effective, less laborious and more accurate when compared with the restriction length fragment polymorphism (RFLP) analysis(Carnevale *et al.,* 2007; LeClair *et al.,* 2013). The LDR-FM technique is deployed to provide an accurate SNPs associated antimalarial drug resistant genes. This method of assay provides high throughput and is suitable for large-scale molecular monitoring and evaluation of *P. falciparum* polymorphisms in malaria endemic settings (Talisuna *et al.,* 2015). This assay method took advantage of the many limitations associated with contamination and decreased efficiency of PCR products analysis. LDR-FMA assays can enable semi-quantitative comparisons between strain-specific polymorphisms (Carnevale *et al.,* 2007) . The LDR mixtures contain an allele specific and common primers. The allelic-specific primers contained a 5' nucleotide sequence and a 3' sequence corresponding to a specific *P. falciparum* polymorphism (LeClair *et al.,* 2013).

2.2.4 Other malaria diagnostic methods

The quantitative buffy coat (QBC) is similar to microscopy of thick blood film. A finger prick blood obtained from an haematocrit tube containing acridine orange and anticoagulant is centrifuged briefly and examined with a UV light enabledmicroscope (Jayakumar, 2011). The nuclei membrane in the parasite fluorescence bright green while the cytoplasm turn to yellow-orange. The sensitivity is almost the same with conventional thick blood film microscopic methods but requires more expensive reagents than microscopy. However, it requires more costly reagents than microscopy with a poor parasite quantification and specie differentiation (Kumar *et al.,* 2017).

2.2.5 Serological malaria diagnosis

Malaria diagnostic by serological method is based on the identification of monoclonal antibodies directed against asexual blood stage of the parasites with an immunofluorescence assay (IFA) (Tangpukdee *et al.,* 2009). Specific antigen are prepared on slides, coated and stored at -30^{O}C. It quantifies both the IgG and IgM antibodies in the serum sample of the patient. Titres >1:20 is considered positive, below 1:20 is considered doubtful or significance while high titres >1:200 indicates recently severeinfection (Chotivanich *et al.,* 2007). Serological technique provides malaria infection retrospectively and is usually deployed for epidemiological surveys.However, the applicability of serological diagnostic method is outdated because of the rigors in antibody

development, its inability to differentiate species andthe requirement for fluorescence (UV) microscope

2.3 Clinical presentation of malaria

2.3.1 Asymptomatic malaria

Individuals with the presence or detection of malaria parasite in blood samples irrespective of its density with an absence of fever and other acute signs and symptoms without recent antimalarial treatment are referred to as having asymptomatic malaria infections (Chen *et al.,* 2016). It poses a major challenge to the ongoing malaria elimination programmes, because these infected individuals usually do not fall sick and so, do not seek treatment which makes them reservoir for further transmission(Ganguly *et al.,* 2013). Significant proportion of malaria infections in endemic areas are sub-patent (sub-microscopic) and are undetectable by conventional diagnostic methods. If untreated, parasite may remain in blood for months to years and may serve as a reservoir for transmission (Bousema *et al.,* 2014). Previously, asymptomatic malaria infection was associated with areas of high transmission, but recent studies from Africa have shown that low-transmission areas are also at the risk of asymptomatic parasitaemia causing approximately 20–60% transmission(Bousema *et al.,* 2014; Okell *et al.,* 2012; Sturrock *et al.,* 2013).

2.3.2 Acute uncomplicated malaria

There are no signs and symptoms peculiar to acute uncomplicated malaria infections. Presenting signs or symptoms are similar to other febrile illnesses. However, the presence of an enlarged spleen (hepatosplenomegaly), thrombocytopenia and anaemia are basically correlated with active malaria infection, especially in young children (Grobusch & Kremsner, 2005). The pre-patent period is the interval between the timesof sporozoites inoculation to erythrocytic stage where parasitesappear in blood sample. The incubation period on the other hand is the interval between infection and clinical appearance of signs and symptoms. Several factors influence the incubation period such as parasite species, transmission intensity, immune status of the host, chemo-prophylactic use of antimalarial drugs, and the parasite density during active infection(Bartoloni & Zammarchi, 2012). Clinical signs and symptoms suggestive of uncomplicated malaria include fever, fatigue, malaise, musculoskeletal pain, anorexia, dizziness, nausea, vomiting with periodic chills and rigor (Bartoloni & Zammarchi, 2012).

2.3.3 Severeand/or complicated malaria

Severe malaria infection is defined as a progression of an acutemalaria with evidence of vital organ dysfunction or/and presence of one or more of severe clinical or laboratory features. Acute uncomplicated malaria can progress to severe malaria if untreated or there is delay in the initiation of appropriate treatment(Kovacs *et al.,* 2015). Cerebral malaria, pulmonary edema, acute renal failure, severe anaemia, as well as bleeding are major signs and complications from severe malaria while acidosis and hypoglycemia are the most widely recognized metabolic complications (Trampuz *et al.,* 2003). If not appropriately treated, any of these complications may progress to death within hours or days. Treatment of severe malaria with prostration, impaired consciousness, convulsions, decreased urinary output, respiratory distress or abnormal bleeding should be treated with parenteral rather than oral antimalarial drugs (Kovacs *et al*., 2015).

2.4 Malaria preventionmechanisms

2.4.1 Vector control

While more resources and interventions has been deployed for effective malaria treatment, an effective preventive measures will reduce the disease prevalence among vulnerable groups in endemic regions if well utilized. WHO recommends the use of vector control or chemoprevention in specific population subgroups via the use of long lasting insecticide treated nets (LLITNs), indoor residual spraying (IRS), intermittent preventive treatment of malaria in pregnancy (IPTp) and seasonal malaria chemoprevention (SMC).

2.4.2 Long lasting insecticide-treated nets (LLINTs)

Long lasting insecticide treated nets (LLITNs) has become the mainstay for vector control in high malaria transmission areas. The effective use of LLITNs have been shown to reduce malaria incidence and death in high transmission settings(Okumu *et al*., 2013).Mass campaigns and public health advocacy sponsored by funding agencies such as Global fund, Bill & Melinda Gates Foundation among others have improved awareness and easy distribution of LLITNs, enabling countries to achieve the universal LLITN coverage. However, there are limited LLITNs to adequately protect all household

members in areas of endemicity. Studies have shown that ownership of LLITNs does not translate to its effective usage(Linn *et al*., 2019; Ruyange *et al*., 2016)

2.4.3 Indoor residual spraying (IRS)

Reducing the mosquito population with indoor residual spraying is an effective strategy in vector control and combatting malaria in areas where the mosquito vectors gain access to living quarters. It is useful as a supplementary tool to long-lasting insecticide-treated net in reducing malaria transmission. The application of IRS to the interior walls of houses is primarily to kill mosquitoes as a vector control method of preventing malaria transmission(Yakob *et al*., 2011)

2.4.4 Malaria during pregnancy

Active malaria infection during pregnancy has contributedsignificantly to maternal morbidity and mortality especially in rural areas where basic health facilities are deficient. Approximately, 125 million women become pregnant in malariaendemic countries yearly and about 30 million live in the tropics where intense transmission ofmalaria occurs all year round(Dellicour *et al.,* 2010). In such areas, malaria infection adversely contribute to maternal and newborn mortality. About 11% neonatal deaths in malaria endemic countriesare attributable to low birth weight arising from *P. falciparum*infections during pregnancy. Intermittent prevention with SP treatment during pregnancy (IPT_P) is effective and has significantly reduced the risk of malaria infection during pregnancy, maternal anaemia, low birth weight, and deaths (Wilson *et al.,* 2011).

2.4.5 Genetically modified *Anopheles* mosquitoes (GMMs)

The historic eradication of malaria in Europe and other regions previously ravaged by malaria was markedly achieved by vector control mechanism via the utilization of insecticides(Takken & Knols, 2009). Recently, genetically modified anopheles mosquitoes (GMM) have been created and they hold a lot of promise in contributing to the goal of malaria eradication and elimination in the nearest future. Genetic engineering technologies can be used both to create transgenic mosquitoes carrying anti-pathogen effector genes targeting human malaria parasites and to generate gene-drive systems capable of introgressing the genes throughout wild vector populations.(Gantz *et al*., 2015).

One of the most successful GMMs method is para-trans-genesis, which involve the genetic manipulation of both commensal and symbiotic bacteria to inhibit the host's capacity to develop and transmit the parasite (Hurwitz *et al.,* 2011). Bacteria are also genetically engineered to secrete peptides and specific proteins that can blockparasite growth and invasion or to completely kill the parasite in the mid-gut. To successfully control malaria with GMM technique, the refractory proteins or peptides are expressed in sufficient amount, the bacteria must target the mid-gut stages of the malaria parasites and maintain their bioactivity in the mid-gut (Riehle *et al.,* 2003). Reports from clinical trials on GMM are quite promising (Spring *et al*., 2013).

2.4.6 Malaria chemoprevention

Following the continuous changes in parasite dynamics andthe transmission pattern of malaria, there is a paradigm shift from the "one size fits all" approach, to a more focusedand targeted malaria control strategies for specific populations and locations for maximal disease containment. Seasonal Malaria Chemoprevention (SMC) has been recommended by WHO to reduce malaria burden in the Sahel region of endemic areas where transmission is predominant in the rainy season(WHO, 2012b). SMC is the intermittent administration of complete course of antimalarial medication to children in areas of high seasonal transmission (Coldiron *et al.,* 2017). This interventional method has shown to be very effective and safe in preventing malaria among children and non-immune adults in low transmission settings (Cissé *et al.,* 2016). A complete dose of amodiaquine plus sulfadoxine-pyrimethamine is the recommended standard for under five years old children at monthly intervals, starting from theonset of transmission season to a maximum of four doses during the malaria transmission season (WHO, 2012b).

2.4.7 Malaria vaccination

The availability of an efficacious malaria vaccines would be a vital addition to the existing armamentarium of anti-malaria tools for the ongoing scaling up efforts towards malaria eradication (Hoffman *et al.,* 2015). Historically, vaccines were instrumental to theeradication of most of the infectious diseases from the Western hemisphere and other parts of the world (Di Pasquale *et al.,* 2015; Smith *et al.,* 2011). Vaccination has become an integral part of managing many infectious diseases. The tremendous successes recorded in previous vaccine application programmes has made it to be considered as the most required preventive medication for effective malaria control, prevention, elimination, and eradication (Hoffman *et al.,* 2015).

However, malaria vaccines development has been a herculean task due to the parasite's complex biological makeup and genetic diversity (Wang *et al.,* 2009). The malaria parasite genome consist of more than 5300 genes with multiple stages of their life cycles compared to viruses and bacteria (Gardner *et al.,* 2002). These different stages of the parasite life cycle are well targeted by the immune system. Cognizance must also be taken of the parasite's location inside or outside of the host cells, and in some cases, these stages are antigenically distinct in their physiological roles (Doolan *et al.,* 2009).

The most widely researched malaria vaccine is on *P. falciparum* which has a complex life cycle with an extensive allelic diversity (Verma *et al.,* 2013). The renewed emphasis is that vaccines for malaria elimination and eradication must be primarily directed towards blocking malaria transmission(Hanboonkunupakarn & White, 2016;Agnandji *et al.,* 2011).

2.5 Malaria chemotherapy

The utilization of antimalarial drugs for chemo-prophylaxis and treatment is fundamental in malaria case management. Several classes of antimalarial drugs are used in the treatment of acute uncomplicated malaria infection. Most antimalarial drugs target the erythrocytic phase of the parasite with gametocidal effects. Unfortunately, drug resistant parasites has emerged to practically all known classes of antimalarial drug (White, 2004), including the ACTs (Hanboonkunupakarn & White, 2016). Artemisinin-based combination therapy is a co-formulated or separately formulated drugs that is taken in combination form, consisting of a rapidly acting artemisinin derivative with very short half-life with a longer-acting (so slowly eliminated) partner drug. The artemisinins rapidly reduces the parasites load and circulating gametocytes that mediate onward malaria transmission while the longer-acting partner drug metabolize more slowly and clears the remaining parasites(Antoine *et al.,* 2014). The short half-life of the artemisinins and the cover provided by the partner drug in the combination therapy provides protection against emergence of parasite resistance to ACTs. The partner drug with prolonged elimination half-life provides post-treatment prophylaxis (Eastman & Fidock, 2009).

The emergence of drug resistance by *P. falciparum* has a correlation with the extensive genetic diversity and parasite dynamics, thus enabling the parasite diverse mechanisms to evade drug therapy (Takala-Harrison & Laufer, 2015; White, 2004). The complex genetic structure of the parasite has also made it difficult in discovering new drug targets and developing novel antimalarial drugs(Gemma *et al.,* 2010).

2.6 Antimalarial drug resistance

The prevalence of chloroquine and sulfadoxine–pyrimethamine (SP) resistance became worsened across malaria endemic regions in the 1990s (White, 2004). This was catastrophic as malaria associated morbidity and mortality rates increased tremendously among African children which accounted for the highest proportion of deaths (Ashley *et al.,* 2014).

Combination therapy as a strategy that has worked to improve treatment outcome and combat resistance of the causative agent which worked in the chemotherapy of tuberculosis, leprosy and HIV was introduced into malaria chemotherapy. Combination therapy can be artemisinin-based or non-artemisinin based (WHO, 2001b). Artemisinin-based combination therapy was the preferred option as the artemisinin component confers the advantage of being gametocidal in addition to its rapid efficacy.Arising from the total failure of chloroquine, SP, and the worsening mefloquine treatment failure, there was an urgent need for effective antimalarial therapy for untreatable malaria cases in Southeast Asia. Parasites in this region has always being the first to develop antimalarial resistance(Amato *et al*., 2018). The World Health Organization (WHO) recommended the use of artemisinin-based combination therapies as first-line antimalarial drugs for *falciparum* malaria (WHO 2001b). The availability and effective use of ACTs, together with the increased utilization of various vector control mechanisms, has tremendously reduced malaria induced morbidity and mortality (Phillips, 2001). However, the recent gains and prospects to eliminate malaria is threatened by the emergence of artemisinin resistant parasites (Dondorp *et al.,* 2009).

Parasite resistance to artemisinin is marked with reduced susceptibility to antimalarial drugs and slow parasite clearance that leads to treatment failure(Ashley *et al.,* 2014). The Kelch 13 gene is implicated as a marker for evaluating artemisinin resistance. Mutations associated at the primary amino-acid sequence of the propeller region is the key causal determinant of artemisinin resistance (Tun *et al.,* 2015).

2.7 Molecular biomarkers of *P. falciparum*

Genetic diversity and routine carriage of multiple allelic genotypes of *P. falciparum* strains are highly prevalent in areas of intense malaria transmission(Niang *et al.,* 2017). The genetic structure and nucleotide variability of *P. falciparum* strains are actively involved in theresponse of the parasite to antimalarial therapies(Congpuong *et al.,* 2014b). Therefore, the genetic diversity of the infecting parasites is an indicator for transmission intensity through the evaluation of the multiplicity of infection (MOI) and to monitor the emergence of parasite resistance (Happi *et al.,* 2009).

The merozoites surface proteins and glutamate-rich protein (*glurp*) are biomarkers used to study the parasite genetic composition, multiplicity of infection, transmission intensity, and the relationship between the host immune system against the parasite (Basco & Ringwald, 2001; Kiwanuka, 2009).

2.7.1*Plasmodium falciparum* chloroquine resistance transportergene

Chloroquine is a diprotic weak base that belongs to the 4-amino quinolone class of antimalarial drugs. It accumulates in the parasite's food vacuole by simple diffusion, trapping and protonation as a result of the acidic nature of the vacuole(Pulcini *et al.,* 2015). It inhibits heme polymerase, the enzyme responsible for heme detoxification in the food vacuole, which becomes toxic to the parasite(Ehlgen *et al.,* 2012). The *Pfcrt* is a membrane-bound integral protein with 10 transmembrane domains (TMD), located on the parasite food vacuole in the membrane of the intra-erythrocytic parasite (Bray *et al.,* 2005; Fidock *et al.,* 2000).*Pfcrt*mutation or polymorphism increases the export of chloroquine from the food vacuole and therefore inhibits heme polymerization. Single nucleotide polymorphisms (SNPs) in *Pfcrt* are baseline biomarkers in assessing chloroquine treatment failure which correlates with resistance (Ecker *et al.,* 2012).

Chloroquine resistance has been strongly linked to polymorphism at position 76 (K76T) in the first TMD of *Pfcrt*(Pulcini *et al.,* 2015). The displacement of amino-acid sequence at this position with a positive charge from asubstrate-binding site in *Pfcrt*, allows the

migration of the protonated out from the food vacuole down its electrochemical gradient (Bray *et al.,* 2005). Mutations at positions K76I and K76N occurs when *P. falciparum* is exposed to lethal concentrations of chloroquine*in vivo*, enhancing parasites survival to drug action(Fidock *et al.,* 2000). The *Pfcrt* function as a chloride channel and proton pump or a regulator of proton pumps. It generally activates or modulates the transport systems in the parasite food vacuole and is being marked as a new drug target (Egan & Kuter, 2013).

2.7.2 *Plasmodium falciparum* multi-drug resistance 1 gene

The *Plasmodium falciparum* multi-drug resistance*1* (*Pf*mrd1) gene, is an orthologue of the mammalian multidrug resistance 1 (MDR1). It was first isolated by S. Foote and collaborators (Foote *et al.,* 1989)and was encoded P-glycoprotein homologue (Pgh). Since its discovery, the *Pfmdr1* gene was observed to be duplicated in some parasites(Das *et al.,* 2014). Increased *Pfmdr1*copy number is linked to parasite responses to a wide range of drugs, and thus, a useful biomarker for multidrug (in contrast to single drug class) resistant malaria (Gil & Krishna, 2017). Increased *Pfmdr1*copy number is especially common in South East Asia, and is positively associated with antimalarial drug resistance(Price *et al.,* 1999, 2006).

The *Pfmdr1* has becomea target gene in monitoring*P. falciparum* resistance to ACTs. A comprehensive knowledge of the gene is essential for epidemiological surveillance and mechanisms to improve resistance-refractory antimalarial drugs development. The unveiling situation of drug collateral sensitivity with specific *Pfmdr1*genetic variation has provided a leadway for personalized optimal therapy (Gil & Krishna, 2017).

2.7.3 Dihydrofolate reductase and dihydropteroate synthase genes

The emergence Sulfadoxine-pyrimethamine (SP) resistant parasites rapidly spread from Southeast Asia to sub Saharan Africa soon after replacing chloroquine as the drug of choice for malaria treatment in the early 1980s (Antony & Parija, 2016; Takala-Harrison & Laufer, 2015). Mutations at specific positions of the *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and *Plasmodium falciparum* dihydropteroate synthase (*Pfdhps*) genes inhibits enzymes that are involved in the folate biosynthesis pathway of the parasite, hence leading to SP resistance (Ndong Ngomo *et al.,* 2016).

Molecular profiling of these mutant genes in parasite populations across malaria endemic regions are well documented (Ojurongbe *et al.,* 2017; Sridaran *et al.,* 2010). Intermediate

resistance has been associated with *Pfdhfr*double point mutation (N51I-S108N and C59R-S108N) while the triple mutation (N51I-C59R-S108N) increases the parasite strain's level of resistance in Africa (Ndong Ngomo *et al.,* 2016; Roper *et al.,* 2003). There also exists a quintuple mutation, a combination of *Pfdhfr* triple mutation (N51I-C59R-S108N) and *Pfdhps* double mutation (437–540 or 437–581), which are the determinants of therapeutic failure to SP (Ndong Ngomo *et al.,* 2016). Several anti-folate drugs are still used in Africa with the same drug targets and mechanism of action. For instance, trimethoprim-sulphamethoxazole drug target as SP in the treatment of bacterial and protozoan infections which may promote cross-resistance to SP which is presently used as chemoprevention of malaria during pregnancy.

Dihydrofolate reductase enzyme is bound by the chlorcycloquanil, the active metabolite of pyrimethamine while sulphadoxine and dapsone are bound by dihydropteroate synthase (Dieckmann & Jung, 1986; Triglia & Cowman, 1999). Tetrahydrofolate production is decreased by the inhibition of dihydrofolate reductase and dihydropteroate synthase, which are enzymes of folate biosynthetic pathway. They serves as a cofactors for the synthesis offolate precursors such as deoxy-thymidine monophosphate (dTMP) and methionine (Ferone, 1977). The *Plasmodium* parasite life cycle is severely inhibited by the reduced synthesis of these precursors (Sibley *et al.,* 2001).

2.7.4 Kelch 13 propeller gene

Occurrence of resistant parasites to artemisinin and its derivatives is of major public health concern. The tremendous gains made in recent times in the global malaria intervention programmes are threatened as a result of the emergence of artemisinin resistant parasite strains. The kelch gene comprises three well defined domains, including:the *Plasmodium* specific domain, BTB/POZ domain, and the kelch propeller domain (Mohon *et al.,* 2014). The kelch propeller domain encodes the kelch13 gene, located on chromosome 13 with 726 amino acid sequence and 83.66 kDa molecular mass.It is the gene of interest widely utilized in the identification artemisinin resistance in clinical isolates(Antony & Parija, 2016; Ariey *et al.,* 2013). Several non-synonymous mutations at the kelch13 propeller domain are linked with an unusual delay in parasite clearance time and reduced survival rates with artemisinin treated patients treated with artemisinin(Antony & Parija, 2016; Ariey *et al.,* 2013).

The WHO defines artemisinin resistance as suspected cases with >5% of patients harbouringthe kelch13single nucleotide polymorphisms (SNPs) while for confirmed

artemisinin resistance the presence of persistent parasitaemia on day 3 or longer parasite clearance time (WHO 2015). The survallance of artemisinin resistance is now focused on the detailed understanding of amino acid sequences of the kelch propeller domain of the K13 gene in *P. falciparum*.

2.8 Allelic diversity of *Plasmodium falciparum*

2.8.1 Merozoite surface proteins

The membrane bound Merozoite surface proteins are abundantly secreted on the merozoite surfaces before reticulocytes and erythrocytes invasion during infection (Beeson *et al.,* 2016). The biological composition and function of these surface proteins are extraordinarily important to malaria researchers. The role they play during RBC invasion, as viable vaccine candidates and as drug targets to inhibit asexual replication during schizogony necessitated their broad investigation (Chandramohanadas *et al.,* 2014; Richards & Beeson, 2009). Relevant proteins involved in the invasion of RBCs are located on the merozoite surface, within the rhoptries and micronemes at the apex of the merozoite. They are either glycophosphatidylinositol (GPI) coupled integral membrane proteinsor as peripheral membrane proteins that are adhesive to the surfaces of the merozoite (Beeson *et al.,* 2016). Some merozoite surface proteins are also localized in the rhoptries and micronemes during schizogony and egress from the schizonts via a variety of mechanisms (Beeson *et al.,* 2016).

The GPI-anchored MSPs mediates the primary attachment of merozoites while subsequent binding is dependent on the erythrocyte binding antigen (EBA) and reticulocyte binding-like homologous (PfRH) proteins(Boyle *et al.,* 2014). The EBA and PfRH ligands are integralmembrane proteins which allows several alternate pathways for merozoite invasion(Tham *et al.,* 2012). Several GPI anchored merozoite surface proteins (MSPs) have been identified (Sanders *et al.,* 2005).

2.8.2 Merozoite surface protein 1

Merozoite Surface Protein 1 (MSP1), also referred as P195, a precursor to the main merozoite surface antigens (PMMSA) or merozoite surface antigen-1(MSA-1). It is the most abundantly expressed GPI-anchored protein (Das *et al.,* 2015; Gilson *et al.,* 2006; Holder *et al.,* 1992). The *msp*1 has a molecular mass of190kD, which undergoes several

proteolytic cleavage prior to merozoite egress from the schizonts(Ekala *et al.,* 2002). The secondary structure of *msp*1 is modified by the cleavage which enhance spectrin binding and mediation of RBC rupture (Das *et al.,* 2015). The proteolytic cleavage processes involved the *msp*1 protein complex to undergo a proteolytic cleavage with four separate polypeptide fragments including the N-terminal fragment consisting 83kD ($msp1_{83}$), two internal fragments consisting 30kD and 38 kDa ($msp1_{30}$&$msp1_{38}$) and the C-terminal with GPI anchored fragment consisting 42 kDa ($msp1_{42}$) with other merozoite proteins like *msp*6 and *msp*7(Holder *et al.,* 1992; Waisberg *et al.,* 2012). During RBC invasion, the$msp1_{42}$ fragment undergoes further cleavages to form two fragments, the $msp1_{19}$ and $msp1_{33}$. The $msp1_{19}$ fragment is attached to the infected RBCs and localizes at the food vacuole during trophozoites formation (Dluzewski *et al.,* 2008).

However, several studies have reported extensive polymorphism associated with *msp*1 gene, making it relatively difficult as transmission blocking vaccine candidate (Atroosh *et al.,* 2015; Chenet *et al.,* 2008; Kang *et al.,* 2010). The *msp*-1 gene has 17 blocks with seven variable regions demarcated by a conserved and a semi-conserved regions. The N-terminal that consists the block-2 region is the most repetitive block with highest genetic recombination and selection within clinical isolates(Holder &Blackman, 1994). Three different allelic families exists in the block 2 region of *msp*1, namely; MAD20, K1 and RO33(Atroosh *et al.,* 2011). The high prevalence of these allelic families are strongly associated with malaria transmission intensity that may induce parasite selection pressure that may cause parasite resistance to antimalarial drugs (Hamid *et al.,* 2013).

2.8.3 Merozoite surface protein-2

The merozoite surface protein-2 (MSP-2) is a glycoprotein that is abundantly expressed on the surface of merozoites with approximately 25 kDa molecular mass(Gilson *et al.,* 2006). The dimorphic*msp*-2 genecomprises two allelic families (3D7 and FC27), consisting N- and C-terminals, with a strain-specific central repetitivedomain (Congpuong *et al.,* 2014a). The 3D7 allele contains of Glycine, Serine and Alanine, while FC27 allelic form contain 32-, 12- and 8-merthionine sequence repeats (Fenton *et al.,* 1991). Natural antibodies expressed against MSP-2 antigens recognizes polymorphic epitopes, and are associated with parasite growth inhibition and conferring clinical immunity to host(Metzger *et al.,* 2003). The *msp*-2 gene is suitable for differentiating genetically distinct *P. falciparum*strains during drug efficacy trial(Ekala *et al.,* 2002).

2.8.4 Glutamate-rich protein

The glutamate-rich protein (*glurp*) has a molecular mass of 220 kDa. It is expressed at pre-erythrocytic and erythrocytic stages of the parasite life cycle, and on the surfaces of newly released merozoites (Borre *et al.,* 1991; Dodoo *et al.,* 2000). It is localized on chromosome 10 and on the *Plasmodium falciparumPf*s38 protein complex on the merozoite surface (Høgh *et al.,* 1993; Pattaradilokrat *et al.,* 2018). The *glurp*gene consists of three well defined allelic domains; a non-repetitive N-terminal (R0), a repetitive central region (R1) and a repetitive C-terminal (R2) (Borre *et al.,* 1991).

The R0 region is anticipated to be hydrophobic and may act as a signal peptide, which is a feature of secretory proteins (Høgh *et al.,* 1993). Antibodies directed towards antibody-dependent cellular inhibition (ADCI) has been linked with antigens released by GLURP in providing acquired protective immunity to malaria infection (Oeuvray *et al.,* 2000). Specific antibodies of GLURP expressed in the systemic circulation are implicated with providing protection against hyper-parasitaemia, the clearance of drug-resistant infections and immunity against disease severity of malarial disease(Enevold *et al.,* 2007; Hogh *et al.,* 1992).

2.8.5 Apical membrane antigen- 1

The apical membrane antigen-1 (AMA-1) is a member of the type-1 integral membrane microneme proteins. Itis involved in forming parts of the moving junction complex that is essential for erythrocyte invasion by merozoites,(Vulliez-Le Normand *et al.,* 2012). Ligands that interfere with the AMA-1 physiologic pathway also inhibit the *in vitro* growth of the asexual blood stages of *P. falciparum*(Lim *et al.,* 2014).

AMA-1 is a promising antigen as malaria vaccine candidate(Miao *et al.,* 2006; Mitchell *et al.,* 2004). It was initially identified in *Plasmodium knowlesi* as a target for monoclonal antibody (mAb) which inhibits erythrocyte invasion and parasite growth (Deans *et al.,* 1982). AMA-1 homologues has been identified in all *Plasmodia species* (Cheng and Saul, 1994; Triglia & Cowman, 1999). Sporozoites are also reported to express AMA-1 and promotes the alteration of hepatocyte invasion by antibodies directed against AMA-1 antigens, suggesting AMA-1 vaccine developed from AMA-1 may be very effective against pre-erythrocytic and erythrocytic*P.falciparum* infection (Li *et al.,* 2002; Malkin *et al.,* 2005; Silvie *et al.,* 2004).

Significant polymorphism has been linked to *Pf*AMA-1 with numerous polymorphic residues at positions within the ectodomain (Chesne-Seck *et al.,* 2005; Coley *et al.,* 2006).

The polymorphic sites are mostly dimorphic and is represented by one or two alternative residual diversity in several positions (Collins *et al.,* 2007). These polymorphisms mostly occur as a result of host immune responses to selective pressure (Coley *et al.,* 2006). The polymorphic residues are usually exposed on the surfaces of the apical membrane the invasion-inhibitory activities of the anti-*Pf*AMA-1 antibody responses are also strain-specificn (Collins *et al.,* 2007; Miura *et al.,* 2007).

2.8.6 Circumsporozoite surface proteins (CSP)

The Circumsporozoite surface proteins (CSP) are polypeptide membrane proteins found on the surfaces of mature and infective sporozoites with an approximately 40-60kDa molecular mass (Fernández-Arias *et al.,* 2015). The CSP was discovered in the 1980s as one of the most abundant proteins on the surface of sporozoite (Nussenzweig & Nussenzweig, 1985). It plays a crucial role during the parasite's life cycle as a multifunctional molecule (Tripathi & Gupta, 2011). The phylogenetic relationship of *Plasmodium* species is defined and characterized by the single-copy CSP gene that encode immunogenic CSP (McCutchan *et al.,* 1996). The CSP has three domains, the N terminus binds to the heparin sulfate proteoglycans, the central repeat domain has four amino acid while the C domain is consist of thrombospondin-like type I repeat (TSR) domain that is actively involved in sporozoite development (Ferguson *et al.,* 2014; Plassmeyer *et al.,* 2009). The central repetitive and C-terminal domains of the *P. falciparum*Circumsporozoite protein are the major target sites for RTS-S vaccine development(Ferguson *et al.,* 2014) and is linked to the hepatitis B surface antigen (Olotu *et al.,* 2014). Monoclonal antibodies raised against CSPs are reported to recognize the immune-dominant repeat domain of CSP and could neutralize parasite infectivity (Cristina *et al.,* 2015).

2.8.7 Erythrocyte binding antigens

The erythrocyte binding antigens (EBAs) is a membrane protein located at the apical domain of the merozoite and are microneme driven. The EBAs families include EBA 175, EBA 140 (BAEBL), EBA 181 (JESEBL) and EBL1 while EBA165 is a pseudogene (Persson *et al.,* 2013; Stubbs *et al.,* 2005, 2005). Each of these genes contains N-terminal. The Duffy binding-like domains, also called F1 and F2 are conserved structures involved in binding RBC receptors(Adams *et al.,* 2001).

Invasion of RBCs by these antigens are mediated by two approaches: the sialic acid (SA) dependent invasion approach with a poor invasion of neuraminidase-treated erythrocytes (neuraminidase cleaves SA on the erythrocyte surface), and the SA-independent invasion approach with a demonstrated efficiency of neuraminidase-treated erythrocytes invasion(Orlandi *et al.,* 1992; Reed *et al.,* 2000). The EBA175 and EBA140 bind to theglycophorin A and C of the erythrocyte surface molecule (Maier *et al.,* 2003; Orlandi *et al.,* 1992). EBA181 is found in chromosome 1 of the *P. falciparum* genome and binds to SA on the erythrocyte surface (Gilberger *et al.,* 2003; Lanzillotti and Coetzer, 2004, 2006). The EBL1 is only expressed by some isolates and binds to glycophorin B (Mayer *et al.,* 2009). The EBAs are also considered as potential vaccine candidates because of their biological role in merozoite invasion and their reduced level of polymorphism (Persson *et al.,* 2013; Richards & Beeson, 2009)

Polymorphisms found in the erythrocyte binding domains are reported to increase parasite survival rate in genetically diverse human populations(Mayer *et al.,* 2004). Antibodies expressed against these antigens induces protective immunity against malaria (Mccarra *et al.,* 2011; Persson *et al.,* 2013; Richards & Beeson, 2009).

2.8.8 *Plasmodium falciparum* microsatellite biomarkers

Microsatellites genes arealso called simple sequence repeats (SSRs) or short tandem repeats (STRs). These are important biomarkers used in *falciparum*population genetic studies. They are short DNA segments containing tandem repeats motifs of about one to six nucleotides and are among the most investigated molecular biomarkers for molecular surveillance of infectious diseases (Guichoux *et al.,* 2011; Orjuela-Sánchez *et al.,* 2013).

Microsatellite genes are abundant in *Plasmodium falciparum* genome sequence with an average of one microsatellite locus in every 2-3 kb. An extensive genetic polymorphism is often associated with microsatellites, resulting to multiple alleles per locus, mostly caused by strand-slippage during DNA replication (Guichoux *et al.,* 2011). The microsatellite approach in molecular analysis such as fingerprinting, parentage analyses, genetic mapping or genetic structure analysis has been used increasingly since in the late eighties(Ellegren, 2004).

2.9 Monitoring of antimalarial drugs resistance

The emergence of resistant parasites to antimalarial drugs poses a major challenge to global efforts on malaria elimination and eradication(Dondorp & Ringwald, 2013; Koukouikila-Koussounda *et al.,* 2017; Miotto *et al.,* 2015; WHO, 2001a). Following the emergence and global spread of chloroquine and sulfadoxine-pyrimethamine resistant parasites (Gatton *et al.,* 2004; Hurwitz *et al.,* 1981; Pillay & Bhoola, 1975), most malaria endemic countries with *P. falciparum* adopted artemisinin-based combination therapy (ACT) as first-line treatment for uncomplicated malaria (Adjuik *et al.,* 2002; Falade *et al.,* 2005; FMoH, 2005; Mårtensson *et al.,* 2005; Staedke *et al.,* 2004). However, the reported occurrence of artemisinin resistance in South East Asia (Imwong *et al.,* 2017; Laufer *et al.,* 2007), calls for a more concerted effort for effective containment of the spread of artemisinin resistance, necessitating a comprehensive antimalarial drug resistance surveillance in curtailing the spread toother regions(Lucchi *et al.,* 2015).

The currently reported *P. falciparum* genes associated with artemisinin resistance are the sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase (SERCA or *PfATPase6*) and Kelch-13 propeller gene (Ariey *et al.,* 2014; Chilongola *et al.,* 2015).

Drug efficacy studies, presence of biomarkers, *in vivo* and pharmacokinetics studies are some methods imploredfor effective surveillance of antimalarial drug resistance(Plowe, 2003).

CHAPTER THREE

3.0 Materials and methods

3.1 Study location and patients

The clinical study was conductedfrom November 2013 to December 2014 in Idi-Ayunre primary healthcare center (rural center), in Oluyole Local Government area (located 35 km away from Ibadan) and a secondary healthcare facility (St. Mary Catholic hospital, Eleta) urban center in Ibadan North-west local government area of Oyo state Ibadan, Southwest Nigeria.

Children of both sex aged 3 to 59 months with complaints of fever (axillary temperature $\geq 37.5^{O}C$) or history of fever in the preceding 48 hours of presentation were enrolled into the study. Enrollees with danger signs of severe malaria, malnutrition and anemia, defined as Hb < 5 g/dl were excluded from the study. A finger prick blood (approximately 50µL)from the middle finger was used to perform RDT, prepare thick blood smears on microscope slides and blood spots on Whartman filter paper (Whartman 3mm™) for PCR procedures.

Malaria RDT screening was performed and read according to the manufacturer's instructions at study sites. Microscopy screening was carried out at the the Institute for Advanced Medical Research and Training (IAMRAT), University College Hospital (UCH) Ibadan while PCR procedures were carried out at the Molecular Biology Laboratory of the Department of Medical Microbiology and Parasitology, College of Health Sciences, Ladoke Akintola University of Technology (LAUTECH) Osogbo, Osun state, Nigeria.

3.2 Study design

A prospective cross sectional and longitudinal study design was employed to evaluate RDT performancein malaria case management as adopted by the National Malaria Treatment Guidelines in Nigeria. Five hundred and eleven (511) children were screened for *falcuparum* malaria with SD-Bioline, an HRP-2 basedmRDT.

3.3 Primary and secondary study outcomes

The primary end pointswere to eveluate the performance of *Pf*HRP-2 based RDTs (SD-Bioline™) in guiding malaria treatment, to evaluate if false-negative RDTs samples are lacking *Pf*hrp-2 and/or *Pf*hrp-3 genes and to identify the nucleotide sequence variation among *Pf*hrp-2 and *Pf*hrp-3 conserved isolates. The secondary outcomes were to evaluate the demographic characteristics of the enrollees with respect to malaria prevalence, analyse the population genetic diversity of allelic frequency distribution of *msp*1, *msp*2 and *glurp* genes and distinguish recrudescence from new infection in recurrence samples during follow up.

3.4 Ethical consideration

The study ethical approval was obtained from the University of Ibadan/University College Hospital Joint Institutional Review Committee (UI/UCH IRC) and Oyo State Ministry of Health IRC boards respectively. A signed informed consent was obtained from each enrollee's parents or guardian before enrollment. Informed consent was obtained in the language the parent/guardian of the prospective enrollees were most comfortable with. Personal information obtained from enrollees during enrolment, examination and follow up was in confidentiality and enrolment into and/or withrawal from the study was absolutely voluntary.

3.5Study inclusion and exclusion criteria

- Children of either sex aged 3 to 59 month old
- Clinical signs and symptoms compatible with acute uncomplicated *falciparum* malaria.
- Axillary temperature ≥ 37.5º C orhistory of fever in the preceding 48 hours.
- Prospective enrollees live within 15km of study locations.
- Willingness to give informed consent and to comply with study procedures.

Enrollees were excluded from the study if one or more of the following symptoms were present:

- Signs and symptoms of complicated or severe malaria.
- Severe malnutrition.

- Severe anaemia with Hb < 5 g/dl.
- Presence of any chronic disease condition. E.g. sickle cell anaemia, renal impairment etc.

3.6 Drug intervention

Children that tested positive to mRDT, recieved weight and age colour coded artesunate/amodiaquine (Winthrop^{Tm} ASAQ) per tablet as stated in Table 3.1.

Table 3.1. Description of ASAQ Doses according to Colour, Body weight and Age of Enrollees

Colour of pack	Weight range	Age range	Artesunate/Amodiaquine content
Pink	4 – 8 Kg	2 – 11 months	25mg/67.5mg
Purple	9 – 17 Kg	1 – 5 years	50mg/135mg
Blue	18 - <36Kg	6 years – 13 years	100mg/270mg

MRDT positive children recieved appropriate doses of ASAQ daily for three days on days 0, 1 and 2 administered by the study nurse. As part of the protocol, all enrollees had a thourough physical examination, blood culture and midstream urine culture to identify co-existing bacteraemia and/or urinary tract infection. Microbiological swabs of the throat and furuncles were also done when indicated. Co-infections and other causes of fever were treated appropriately.

3.7 Quality control and assurance procedures

The study was conducted in accordance with Good clinical Practice (GCP) and good laboratory practice. GCP defined as an international standard for the design, conduct, performance, monitoring, auditing, recording, analyses, and reporting of clinical trials while GLP is defined as standard guidelines for proper operation of equipment, maintenance and sanitation, reporting structures, biohazard and safety measures(Adamo *et al*., 2012; Arango *et al*., 2016; Bornstein-Forst, 2017). The safety of all enrollees were considered a top priority to the research team throughout the study period. Adverse events

were attended to promptly. The RDT readings were carried out by two research assistants while discordant results were read by the principal investigator as final judgement. Results of RDT was blinded to microscopy while both RDT and microscopy results were blinded to PCR results to avoid bias judgement.

A laboratory cultured 3D7 *P. falciparum* strain spotted on DBS was used as positive control for parasite speciation, *msp*1, *msp*2 and *glurp* genotyping while PCR grade nuclease free water was used as negative control.

3.8.0 Data collection

3.8.1Demographic and laboratory data collection procedures

A structured Case Record Form (CRF) was used to collect data on demographic, socio-economic characteristics, and history of current and past illnesses as well as treatments received at home and from other health facilities before consultation at the study clinic. Children were examined by a medical officer and relevant clinical findings were documented. Other relevant biological samples taken from the participants included: thick blood smears for malaria microscopy, blood spot on filter paper and other causes of fever investigations.

3.8.2 Malaria rapid diagnostic tests (mRDTs)

The *Pf*HRP-*2* based RDT brandevaluated during this study was SD-Bioline^{TM} (Standard Diagnostic, Inc. Korea). Malaria rapid diagnostic test was conducted and read in accordance with manufacturer's guidlines. Briefly, The pulp of the left middle finger was cleaned with alcohol pads until there was novisible stain on the alcohol pad and then allowed to dry. Capillary blood was obtained after pricking with a sterile lancet. Five microliter (5µL) of blood was taken from the drop of capillary blood with the aid of the disposable loop provided and placed in the round sample well of a diskette on a flat horizontal table away from sunlight. Four drops of the buffer was added into the square assay diluent well. The diskette was read after 15minutes and if negative, re-read after another 10 to 15 minutes as per the instructions of the manufacturer. Results were read by two independent research assistants. The appearance of a "C"(control) band or line in the result window was considered negative, two bands or lines "C" i.e. ''C'' (control line) and "T" (test line) in the result window was considered positive while diskettes without a "C" (control) line was considered invalid.

3.8.3 Microscopy malaria diagnosis

Capilary blood from finger prick was collected at presentation to prepare thick blood film and was stained with freshly prepared 10% Giemsa solution at pH 7.2 following standard proceedure (Fleischer, 2004). The thick films for parasite detection and quantification were screened by two independent expert microscopists blinded to RDT results at the Institute for Advanced Medical Research and Training (IAMRAT), University College Hospital, Ibadan. Microscopy readings were considered negative if assexual parasites (trophozoites and schzonts) was not detected after viewing 100 high power fields. Parasite density was calculated based on the number of asexual parasites against about 200 leukocytes (WBCs) and multiplying with an assumed leukocyte count of 8,000/mm^3 (Trape, 1985).

3.8.4Sample collection for molecular biology

Samples for molecular assays was collected on Whatman 3mm^{TM}filter paper. Dried blood spots (DBS)was prepared from finger prick blood and air-dried at room temperature. Each DBS was wrapped in a plastic zip-lock sample bag with desicants to avoid sample contamination. The packaged DBS were transported to the molecular biology laboratory of the Department of Medical Microbiology, College of Health Sciences, Ladoke Akintola University of Technology (LAUTECH) Osogbo for molecular biology procedures.

3.8.5 Genomic DNA extraction procedures

Genomic DNA was extracted from DBS with QIAamp DNA Mini kit blood and tissue (QIAGEN Germany) according to the manufacturers instructions as follows:

1. With a sterilized paper puncher, about 3 punched-out circles of DBS was added into a 1.5mL micro-centrifuge tube with 180µL cell/tissue lysis buffer ATL.

2.Samples were incubated in an electrical heating block at 85^{o}Cfor 10 min and was briefly centrifuged to remove steam from inside the lid after incubation.

3.20µL proteinase K stock solution was added, briefly vortex rigorously and incubated at 56^{o}C for 1h. Centrifuge to remove steam from inside the lid.

4.200µL of buffer AL (tissue stabilizing butter to stop destruction of DNA) was added to the sample.Vortex thoroughly and incubated at 70^{o}C for 10 min. Centrifuge briefly to remove steam from inside the lid.

5.Add 200µL ethanol (96 – 100% concentration) to sample and vortex vigorously. Briefly centrifuge to remove drops from inside the lid.

6.The content in step 5 above was carefully transferred into QIAamp mini spin column with a collection tube andwas centrifuged at 6000 x g (8000 rmp) for 1 min. The tube containing the filtrate was discarded while the QIAamp mini spin column was placed in another clean collection tube.

7.500µL wash buffer AW1 was added and was centrifuged at 6000 x g (8000 rmp) for 1 min. The collection tube containing the filtrate was discarded and the QIAamp mini spin column was placed in another clean collection tube.

8.500µL wash buffer AW2 was added and centrifuged at full speed 20,000 x g(14,000 rmp) for 3 min.

9.The QIAamp Mini spin column was placed in a new 2mL collection tube and the old collection tube was discarded with the filtrate. The QIAamp Mini column was centrifuged at full speed for 1 min to remove carryover AW2 wash buffer.

10. The QIAamp Mini spin column was placed in a clean 1.5µL microcentrifuge tube and the collection tube containing the filtrate was discarded. 80µL of elution buffer AE was added and incubated at room temperature (15 – 20^{o}C) for 1 min, and centrifuge at 6000 x g (8000 rpm) for 1 min. The extracted DNA was stored at – 20^{o}C until further PCR analysis.

3.9 PCR Amplification and speciation of *Plasmodium* Using18SrRNA gene

In a nested PCR amplification protocol, a primary reaction targeting the 18SrRNA gene of *Plasmodium* as previously described (Snounou *et al.,* 1993) was amplified. The genus-specific primers sequences were rPLU5 (5'CCTGTTGTTGCCTTAAACTT 3') as forward primer and rPLU6 (5'TTAAATTGTTGCAGTTAAAACG 3') as the reverse primer (see appendix 1). In a 20µL reaction volume, PCR mixture containing 2µL of 10X PCR buffer, 25mM $MgCl_2$, 10mM dNTPs (New England Biolabs, UK), 0.5mM each of primers (forward and reverse), 0.2U Taq polymerase (One-Taq polymerase, New England Biolabs UK), 10.8µL nuclease free PCR grade water and 5µL DNA template was prepared for the primary reaction.

Similarly, a second set of specie-specfic primer pairs for *P. falciparum, P. ovale, P. malariae* and *P. vivax*was amplified procedurally as previously described (Snounou et al 1993). In the Nested reaction, 2µL of the primary PCR product was used as template in a reaction volume of 20µL containing 2µL of 10X PCR buffer, 25mM $MgCl_2$, 10mM dNTPs (New England Biolabs, UK), 0.5mM each of primers (forward and reverse), 0.2U

Taq polymerase (One-Taq polymerase, New England Biolabs UK) and 13.8µL nuclease free PCR grade water. The expected amplicom size for *P. falciparum* (205bp), *P. ovale*(800bp), *P. malariae*(145bp)and *P. vivax*(117bp) respectively.

3.9.1 Primary PCR cycling conditions for *Plasmodium* 18SrRNA gene amplification

Initial denaturation	95^{O}C	5min	1 cycle
Denaturation	94^{O}C	1min	
Annealing	58^{O}C	2min	25 cycles
Extension	72^{O}C	2min	
Final extension	72^{O}C	5min	1 cycle
Final hold	10^{O}C		infinity

3.9.2 Nested (Secondary) PCR cycling conditions for *Plasmodium spp*. amplification

Initial denaturation	95^{O}C	5min	1 cycle
Denaturation	94^{O}C	1min	
Annealing	58^{O}C	2min	30 cycles
Extension	72^{O}C	2min	
Final extension	72^{O}C	5min	1 cycle
Final hold	10^{O}C		infinity

3.10 Analysis of the performance of SD-Bioline-*Pf* malaria RDT

All children that met the study enrolment criteria were screened with SD-Bioline *Pf*HRP-2 specific RDT, thick blood smears were prepared for microscopy and DBS obtained for molecular detection and speciation of malaria parasite. The performace of SD-Bioline and PCR was compared with microscopy as gold standard. The diagnostic outcome was compared by calculating sensitivity and specificity through the number of true positive (TP), true negative (TN), false positive (FP) and false negative (FN) cases as detected by RDT. Malaria RDT results were considered true positives (TP) or true negatives (TN) if they were concordant with microscopy or PCR results depending on the test regarded as gold standard. Negative RDT were considered false-negatives (FN) if microscopy or PCR was positive. Positive RDT results were considered false positives (FP), if microscopy or PCR was negative. RDT performance was evaluated by calculating the sensitivity, defined the probability that a test result will be positive when the disease is present (true positive

rate),using the mathematical formula TP/TP + FN. Specificity was defined as theprobability that a test result will be negative when the disease is not present (true negative rate), calculated as; TN/TN + FP, positive predictive value (PPV) was defined as the likelihood that the disease is present when the test is positive (TP/TP + FP) and negative predictive value (NPV), defined as the likelihood that the disease is not present when the test is negative (TN/FN + TN). Test accuracy, which is defined as the proportion of all tests that gives correct results was calculated as (TP + TN/TP + TN + FP + FN) (Baratloo *et al.,* 2015).

The measure of agreement between the diagnostic methods was assessed by calculating the kappa statistics (k) and corresponding standard error of mean (Gatti *et al.,* 2007; Landis &Koch, 1977); k-value < 0.00 = poor agreement, 0.00 – 0.20 = slight agreement, 0.21 – 0.40 = fair agreement, 0.41- 0.60 = moderate agreement, 0.61 - > 0.80 = substantial agreement while 0.81 – 1.00 was considered almost perfect agreement.

3.11 Evaluation of *Pf*hrp-2/*Pf*hrp-3 deletion on RDT false- negative samples

In a semi-nested PCR reaction, the exon 2 regions of *Pf*hrp-2 and *Pf*hrp-3 genes were amplified using primer sequences and PCR protocols as previously described (Baker *et al.,* 2005) with slight modification on the number of PCR cycling conditions. The PCR assay was to determine the presence or absence of *Pf*hrp-2 and/or *Pf*hrp-3 gene deletions in the RDT false negative samples.

In the primary reactions, 20µL PCR mix containing 2µL 10X PCR buffer, 25mM Mg2Cl, 10mM each dNTPs (Bioline UK), 0.5mM of each primer, 0.3U of FIREpol Taq (Bioline UK), 10.8µL of nuclease free PCR grade water and 5µL of DNA as template.

In the semi-nested reactions, a final reaction volume of 20µL containing 2µL of 10X PCR buffer, 25mM Mg2Cl, 10mM each dNTPs (Bioline UK), 0.5mM of each primer, 0.3U of FIREpol Taq (Bioline UK), 13.8µL of nuclease free PCR grade water and 2µL of the primary PCR product as a DNA template.

In each of the PCR runs, three set of controls were added. An*In-vitro* cultured *P. falciparum*3D7 was used as positive control for *Pf*hrp-2 and *Pf*hrp-3, a known hrp-2 deleted Dd2 (*Pf*hrp-2 negative) as negative control for hrp2 and a known hrp3 deleted Hb3 (*Pf*hrp-3 negative) as hrp-3 negative control. The expected fragment sizes for both *Pf*hrp-2 and *Pf*hrp2 range from 500 to 900 bp.

3.11.1 Semi Nested PCR cycling conditions for *Plasmodium falciparum* hrp2/3 genes

Initial denaturation	94^{o}C - 5 min	1 cycle
Denaturation	94^{o}C - 50s	27cycles for primary reaction and 32 cycles for semi-nested reaction
Annealing	55^{o}C - 50s	
Extension	72^{o}C - 1min	
Final extension	72^{o}C 10 min	1 cycle
Hold	10^{O}C	---

3.12 Analysis of recrudescence and re-infection following ASAQ treatment

29 of 205 (12.7%) of the microscopically confirmed malaria positive enrollees had recurrent parasitaemia post-treatment.To validate the therapeutic efficacy of artesunate/amodiaquine, paired DNA samples from pre-treatment (day 0) and any other day of recurrent infection during the 28 days follow up period was analysed by genotyping*msp*2 and *glurp* genes to distinguish new infections (defined as recurrent infection between day 14 and 28, post-treatment in areas of high transmission due to exposure to new mosquitoes bite)and recrudescence infections (defined as the early re-emergence of uncleared asexual parasitaemia within day 3 to day 7 following antimalarial treatment, which is a function of the susceptibility of the parasite and the elimination kinetics of the antimalarial drug)(Padalia & Modi, 2009).

3.13 Allelic Genotyping of *P. falciparummsp*1, *msp*2 &*glurp* Genes

Specific oligos primers targeting the polymorphic regions of *msp*1and *msp*2 (block 2 & 3) (Snounou *et al* 1993) and *glurp* R2 region were used for allelic genotyping and chracterization. The antigenic markers of *msp*1 (K1, MAD20, & RO33) and *msp*2 (FC27 & 3D7) were amplified. In a final reaction volume of 20µL, containing 10X PCR buffer 2µL, primers (forward and reverse) 0.8mM each, 25mM $MgCl_2$, dNTPs 10mM, 0.2U

oneTaq polymerase (New England Biolabs. Inc. UK) and 5µl of DNA template in the first/primary reaction and 2µL of the primary PCR product as template in the secondary (nested) reaction. Expected amplicom sizes for *msp*1 range from 150- 600 bp, *msp*2 200 – 900bp and *glurp* 500 – 1200 bp respectively. The PCR cycling conditions for both primary and nested reactions are as previously described (Snounou *et al.,* 1999).

Aprimary and semi-nested PCR amplification in a final reaction volume of 25µL for the polymorphic R2 region of *glurp* was performed in a reaction mixture containing 2.5µL 10X PCR buffer, 25mM $MgCl_2$, 10mM dNTPs, 0.75mM oligos each (forward and reverse), 0.2U OneTaq polymerase and 5µL of DNA template in the primary reaction while 2µL primary PCR products was used as the DNA template for the semi-nested reaction with the same conditions.

3.13.1 PCR cycling conditions for *msp*1 and *msp*2 genes amplification

Initial denaturation	$94^{O}C$	- 5min	1 cycle
Denaturation	$94^{O}C$	- 10s	30 cycles Primary and Nested reactions
Annealing	$57^{O}C$	- 30s	
Extension	$72^{O}C$	- 40s	
Final Extension	$72^{O}C$	- 3min	1 cycle
Hold		$10^{O}C$	infinity

3.13.2 Primary PCR cycling conditions for *glurp* gene amplification

Initial denaturation	$94^{O}C$	- 2min	1 cycle	
Denaturation	$94^{O}C$	- 30s		
Annealing	$54^{O}C$	- 1min	30 cycles	Primary reaction
Extension	$72^{O}C$	- 1min		
Final Extension	$72^{O}C$	- 5min	1 cycle	
Hold		$10^{O}C$	infinity	

3.13.3 Semi-nested PCR cycling conditions for *glurp* Gene amplification

Initial denaturation	$94^{O}C$	2min	1cycle	
Denaturation	$94^{O}C$	-1min		
Annealing	$59^{O}C$	-1min	30 cycles	Semi- nested reaction
Extension	$72^{O}C$	1min		
Final Extension	$72^{O}C$	5min	1 cycle	
Hold	$10^{O}C$		infinity	

3.14Sequencing of conserved region of*Pf*hrp-2 and *Pf*hrp-3 isolates

Isolates that were successfully amplified for the exon-2 region of *Pf*hrp-2 and *Pf*hrp-3 genes were further sequenced to elucidate the nucleotide sequence variability of *Pf*hrp-2 and *Pf*hrp-3 genes.The *Pf*hrp2 and *Pf*hrp3 amplified PCR products were purified using Exo-SAP-IT PCR Product Cleanup Reagent (USB, Affymetrix, Santa Clara, CA), according to manufacturer's instructions. Purified PCR products were sequenced at the Institute of Tropical Medicine Tuebingen, Germany using the Big Dye Terminator v. 2.0 cycle sequencing kit (Applied Bio-systems, Beverly, MA) according to the manufacturer's instructions. BioEdit was used to assemble nucleotide sequences and amino acid sequences of *Pf*hrp-2 and *Pf*hrp-3 were deduced using ExPASy translate tool. Differences in the number of repeats of each type and the total number of repeats were tested by logistic regression.Amino acid repeat sequences were identified and given appropriate numeric codes ranging from type 1 to type 20 as reported previously (Baker *et al*., 2005; Bharti *et al.*, 2016).

3.15 Gel electrophoresis of PCR products

After the nested or secondary PCR amplification, the amplicom (products) were run on a 1.5% agarose. About 5µL of theamplicom was mixed with 5µL loading buffer containing glycerol plus SYBR-Green® (Cambrex Biosciences, East Rutherford, NJ, USA). The loaded PCR products were allowed to migrate freely in an electrophoretic tank with positive and negative electrical charges at 100-volt. Since DNA is negatively charged, amplified genes migrated towards the positive pole. After 40 to 60min, accumulation of the same fragment sizes binds together and formed a clear band separation according to base pair (bp) sizes. The gel was placed under a 312 nm UV trans-illuminator, visualized and documented using an electronic photographic documentation system. *Plasmodium species* differentiation was documented according to fragment sizes (*P. falciparum* 205bp, *P. ovale* 800pb, *P. malariae* 145bp and *P. vivax* 117 bp).

3.16 Data management and analysis

Data was seperately entered into Microsoft Excel spreadsheet and IBM SPSS (version 20) for statistical analysis. The RDT performance and PCR detection rate was eveluated by calculating the sensitivity, specificity, positive and negative predictive values against microscopy (thick smears) as gold standard.EpiCalc (version 2000) was used for statistical extrapolation.

The level of agreement between the diagnostic methods was assessed by calculating the κappa-statistics (κ). A κ-value < 0.00 was taken as poor agreement, 0.00 – 0.20was considered slightagreement, 0.21 – 0.40as fairagreement, 0.41- 0.60 as moderate agreement, 0.61-0.80 as substantialagreement and 0.81 – 1.00 as almostperfect agreement(Gatti *et al.,* 2007; Landis & Koch, 1977).

The *msp*1, *msp*2 and *glurp* allelic frequencies were evaluated by calculating the proportion of alleles detected in a particular allelic family out of the alleles amplified in the isolates. Isolates with multiplefragments sizes was considered infections with multiple parasite strains, categorized as frequency of multiplicity of infection (MOI). The mean multiplicity of infection (MOI) was calculated as a total number of *P. falciparum*(*msp1, msp2 and glurp*) genotypes detected per total number of positive samples. Data was analyzed using descriptive statistic, paired student t-test and Chi-square. Statistical significance was set at p-value ≤ 0.05.

CHAPTER FOUR

Results

4.1 Demographic profiles of study enrollees

The study was conducted in a rural primary healthcare and secondary health facilities in Ibadan, southwest Nigeria from November 2013 to December 2014. A prospective cross sectional and longitudinal study design was carried out to evaluated the diagnostic performance of malaria rapid diagnostic tests, PCR speciation of prevalent parasite species, investigation of RDT false negative results for*Pf*hrp2/3 gene deletion, discrimination of new from recrudescence infections following ASAQ treatment and *Pf*hrp2/3 nucleotide variability and the allelic frequency distribution of *msp1*, *msp2* and *glurp* genes in the parasite sub population were evaluated.

A total 511 febrile children were enrolled after consent was voluntarily given by parents/guardians to participate in the study. Blood samples for malaria screening was collected by finger prick for malaria RDTs, thick blood films for microscopy and DBS for molecular biology procedures.

Out of this number, 485/511 (94.9%) children had a complete RDT, microscopy and PCR samples that was successfully analyzed. Of the 485 children, 340 (70.1%) and 145 (29.9%)were enrolled at the rural and urban sites respectively. The mean age (months) of the enrollees was 26.6 ± 15.7 while the mean temperature was 37.7^{o}C ± 1.2 (range 35.6 – 40.7^{o}C). Details of the demographic and clinical characteristics of the study participants are shown on table 4.1.

Table 4.1. Clinical and Demographic Characteristics of Study Participants

Characteristics	Total N = 485 (%)	Rural N = 340 (%)	Urban N = 145 (%)	p- value
Gender				
Male	289 (59.6%)	204 (60.0%)	85 (58.6%)	0.427
Female	196 (40.4%)	136 (40.0%)	60 (41.4%)	
Age (months)				
Mean ± SD	26.6 ± 15.7	26.6 ± 15.5	26.7 ± 16.0	0.931
Range	3-59	3-59	3-59	
Weight (kg)				
Mean ± SD	11.3 ± 3.2	11.1 ± 3.2	11.6 ± 3.3	0.253
Range	4.8 - 27	4.8 - 27	5.4 21.9	
Height(cm)				
Mean ± SD	81.1 ± 16.1	82.5 ± 14.0	77.6 ± 19.8	0.002
Range	23.5 - 115.3	24.0 - 115.3	23.5 - 110.0	
Temp.(^{o}C)				
Mean ± SD	37.7 ± 1.2	37.8 ± 1.2	37.3 ± 1.1	0.546
Range	35.6 - 40.7	35 - 40.7	35.2 - 40.5	
Temp. ≥ 37.4^{o}C	235 (48.5%)	185 (54.4%)	50 (34.5%)	< 0.001
Baseline PCV (%)				
Mean ± SD	31.7 ± 4.6	31.3 ± 4.8	32.5 ± 4.0	0.007
Range	17 – 45	17 – 45	19 - 45	

PCV = Packed Cell Volume, SD = Standard Deviation

4.2 Malaria parasite detection by RDT (SD-Bioline™), microscopy and PCR

The prevalence of *P. falciparum* detected by mRDT (SD-Bioline™), microscopy and PCR was 59.6% (289/485), 43.5% (205/485) and 51.3% (249/485) respectively. The rural site recorded a higher prevalence of 67.6%, 50.3% and 60.9% compared to 40.7%, 23.4% and 29.0% recorded in the urban site for RDT, microscopy and PCR respectively. The difference in malaria prevalence between the rural and urban sites based on the diagnostic methods was statistically significant ($p<0.001$) (Table 4.2). PCR performance was the most sensitive followed by RDTs while the specificity was higher in PCR and microscopy than RDT. The geometric mean parasite density was higher among rural malaria infected children compared to those at the urban study site but the difference was not statistically significant ($p=0.527$). *Plasmodium falciparum* (91.6%; 228/249) was the commonest specie detected by PCR during speciation in both sites followed by *P. malariae*, (18.9%; 47/249) and *P. ovale (*4.4%; 11/249). No case of *P. vivax* was detected. *Plasmodium malariae and P. ovale* mostly occurred as mixed infections with *P. falciparum* (Table 4.2). There was a significant difference observed in the prevalence of mixed infections of*P. falciparum* + *P. malariae* ($p<0.001$) and *P. falciparum* + *P. ovale* ($p= 0.016$) between the rural and the urban site with the rural site recording the highest prevalence for both co-infections (Table 4.2). PCR fragment sizes of *P. falciparum*, *P. malariae* and *P. ovale* are shown in figures 4.1, 4.2 and 4.3 respectively.

Table 4.2. Malaria Parasite Detection Rate by RDT (SD-Bioline), Microscopy and PCR

Variable	Sample size (N = 485)	Idi-ayunre (N = 314)	Eleta (N = 171)	P- value
SD-Bioline	289 (59.6%)	230 (67.6%)	59 (40.7%)	< 0.001
Microscopy	205 (43.5%)	171(50.3%)	34 (23.4%)	< 0.001
PCR	249(51.3%)	207 (60.9%)	42 (29.0%)	< 0.001
Parasite Density (µL)				
Mean	41,099	42,550	33,963	0.527
GEOMEAN	8,012	9,131	3,598	
Range	28 - 611,600	28 - 611,600	40 - 311,200	
***Plasmodium* speciation**				
P. falciparum	228 (91.6%)	193 (93.2%)	35 (83.3%)	< 0.001
P. malariae	47 (18.9%)	39 (18.8%)	8 (19.0%)	0.978
P. ovale	11 (4.4%)	11 (5.7%)	0 (%)	0.144
P. f + P. m	31/47 (66.0%)	30/39 (76.9%)	1/39 (2.6%)	< 0.001
P. f + P. o	6/11 (54.5%)	6/11(54.5%)	0 (0%)	0.016

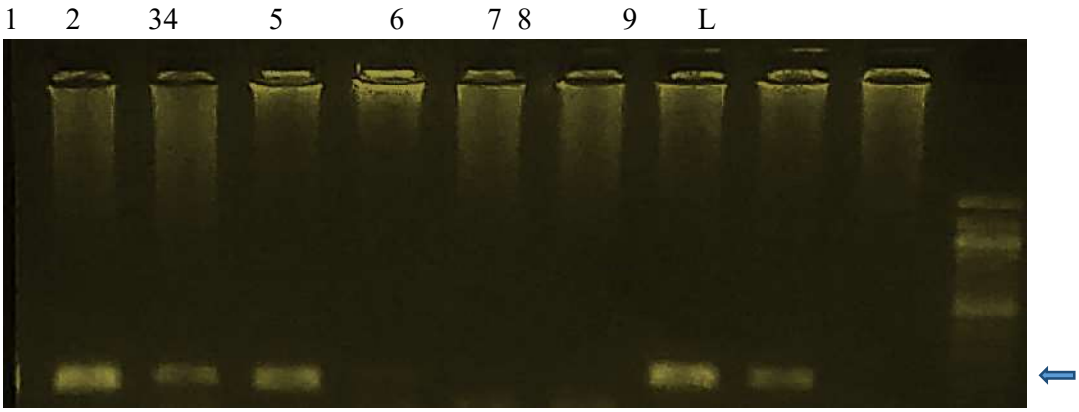


Fig 4.1. *P. falciparum* Gel electrophoresis

L = 100bp DNA molecular marker,

Well 9 Negative control,

Well 1 = Positive sample

Well 2 – 8 = Samples

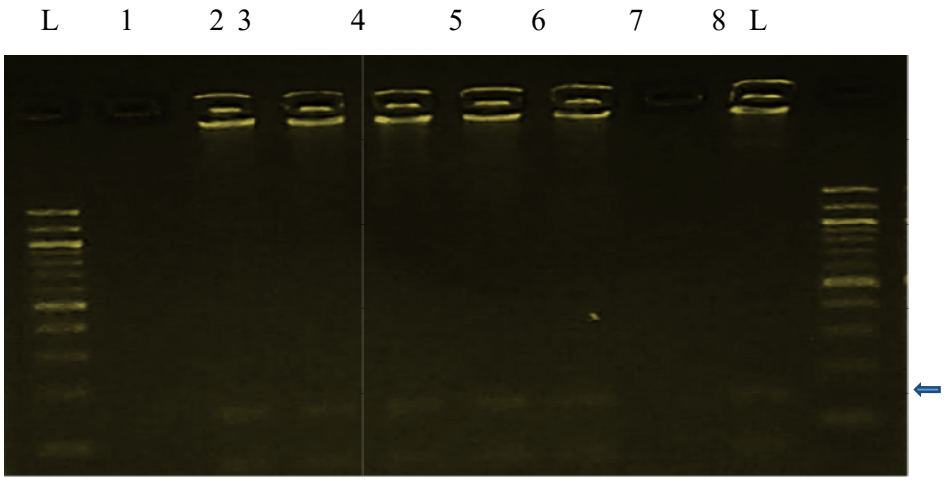


Fig 4.2. *P. malariae* Gel electrophoresis:

L=100bp DNA molecular marker,

Well 8= Positive control,

Well 2 – 7 Samples

Well 1= Negative control

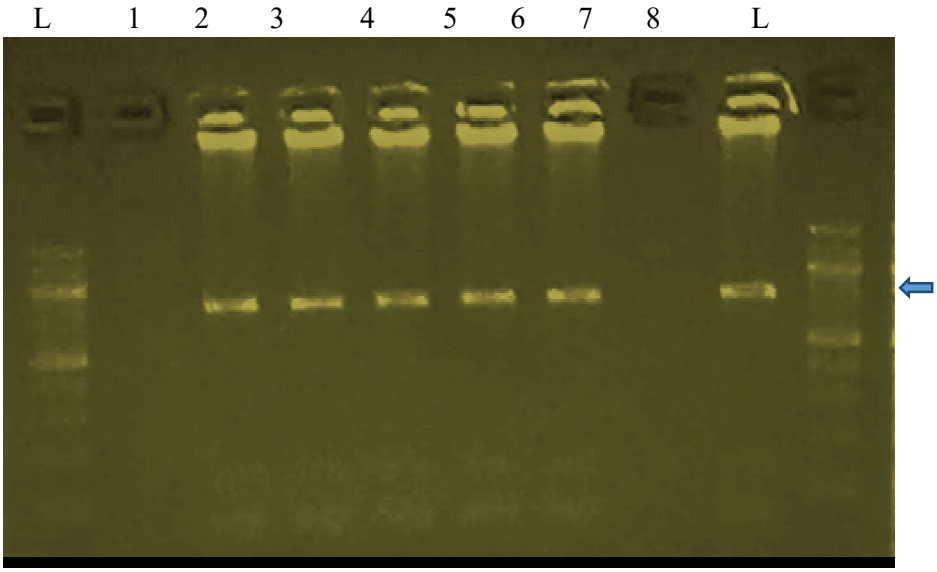


Fig 4.3. *P. ovale* Gel electrophoresis:

L = 100bp DNA molecular ladder,

Well 8 = Positive control,

Well 1 = Negative control,

Wells 2 – 7 Samples

4.3 Diagnostic performance of RDT using microscopy and PCR as gold standard

The diagnostic performance of RDT was evaluated using microscopy and PCR as the gold standard. Overall, 289/485 (59.6%) participants tested positive to RDT (SD-Bioline) with 4.2% (12/280) cases of false negative and 43.9% (90/205) false positive against microscopy as gold standard, giving a sensitivity of 95%, specificity of 67, negative and positive predictive values of 94% and 68% respectively. There was a decrease in RDT performance when compared with PCR as the gold standard. Thirty nine 16.5% (39/236 false negative and 31.7% (79/249) false positive results were recorded. The sensitivity was 84%, specificity was the same 67%. Accuracy was 76% while negative and positive predictive values were 80% and 73% respectively. The κ-statistics was 0.583 when RDT was compared with microscopy and 0.511 for PCR. More details on the performance of SD-Bioline against microscopy and PCR as gold standards are shown (Table 4.3).

Table 4.3. Comparison of RDT (SD-Bioline) Performance using Microscopy and PCR as Gold Standard

Variable	RDT/Microscopy (%)	CI (%)	RDT/PCR (%)	CI (%)
Prevalence	43	38 - 47	51	47 – 56
Sensitivity	95	90 - 97	84	79 – 89
Specificity	67	61 - 73	67	60 – 72
Accuracy	79	75 - 82	76	72 – 79
Negative predictive value	94	89 - 97	80	74 – 85
Positive predictive value	68	62 - 73	73	67 – 78
k- statistics	**0.583**		**0.511**	

4.4 Diagnostic performance of RDT and microscopy against PCR as gold standard

Similarly, the performance of RDT (SD-Bioline) and microscopy was compared with PCR, sensitivity was higher with RDT (84%) while microscopy sensitivity was lower (75%). The reverse was the case for specificity as RDT was 67% while microscopy was 93%. The kappa's level of agreement between RDT/PCR was 0.511 while microscopy/PCR was 0.688. Microscopy had a substantial agreement with PCR while RDT had a moderate kappa's value Further details are shown on Table 4.4.

Table 4.4. Comparison of SD-Bioline and Microscopy using PCR as Gold Standard

Variable	**RDT /PCR (%)**	**CI (%)**	**Microscopy/PCR**	**CI (%)**
Prevalence	51	47 - 56	51	47 - 56
Sensitivity	84	79 - 89	75	71 - 82
Specificity	67	60 - 72	93	88 - 95
Accuracy	76	72 - 79	84	81 - 87
Negative Predictive Value	80	74 - 85	79	86 - 94
Positive Predictive Value	73	67 - 78	92	74 - 84
K-Statistics	**0.511**		**0.688**	

4.5 Risk of anaemia among mRDT positive versus mRDT negative Enrolees at baseline and post treatment

The proportion of children with anaemia (haematocrit < 30%) was higher in the mRDT positive compared to mRDT negative children at enrolment (37.9 vs 10.0%); $p < 0.001$). The proportion of children having anaemia at day 28 post treatment decreased in both mRDT positive and negative children. However, a higher proportion of mRDT positive children was anaemic compared to mRDT negative children at day 28 post treatment (12.6 vs 3.1%; $p = 0.001$) (Table 4.5)

Table 4.5. Risk of Anaemia among Febrile under 5 year's old children in Ibadan, Nigeria

Characteristic	MRDT-positive on day 0	MRDT-negative on day 0	ρ-value
Anaemia day 0	116/306 (37.9%)	20/200 (10.0%)	<0.0001
Anaemia Day 28	29/231 (12.6%)	5/160 (3.1%)	0.001

*Using microscopy Anaemia was defined as haematocrit less than 30%

4.6 Detection of *Pf*hrp-2 and *Pf*hrp-3 genes deletion

The extent of *Pf*hrp-2/*Pf*hrp-3gene deletionwas investigated usingsixty six (66) samples comprising of RDT false negative (31) and true positive (35). The presence or absence of *Pf*hrp-2 and/or *Pf*hrp-3 genes were confirmed using a semi-nested PCR method targeting the exon-2 region. The amplicom size for both *Pf*hrp-2 and *Pf*hrp-3 genes range from 500 to 900 base pair.The overall results show that out of the 66 samples that were amplified, 11 (16.7%) were observed to have the *Pf*hrp-2 gene deleted while 4 (6.0%) had*Pf*hrp-3 gene deletion. Among the 31 samples with RDT false negative, 8 (25.8%) had *Pf*hrp-2 gene deletion while 4 (12.9%) had *Pf*hrp-3 gene deletion. Of the 11 isolates that had *Pf*hrp-2 deletion, 7 isolate had a conserved *Pf*hrp-3 exon-2 region. Three *Pf*hrp-3 conserved antigens cross reacted with the *Pf*HRP-2 based RDT to give positive results even though the exon-2 region of *Pf*hrp-2 gene was deleted in those samples (Tables 4.6a &b, Figures 4.4 & 4.5).

Table 4.6a. Distribution of *Pf*hrp2 and *Pf*hrp3 Deletions in*Plasmodium falciparum* Isolates

Variable	No. of Isolates	*Pf*hrp-2 Deletion	*Pf*hrp-3 Deletion
RDT False -ves and True +ves	66	11(16.7 %)	4(6.0 %)
RDT False –ves	31	8(25.8 %)	4(12.9 %)
RDT True +ves	35	3 (8.6 %)	0 (0 %)

Table 4.6b.*Pf*HRP-2 Based RDT Cross Reactivity with *Pf*HRP-3 Antigen

Variable	No. of Isolates	Percentage(%)
*Pf*hrp-2/ *Pf*hrp-3 conserved Isolates	55/66	83.3
*Pf*HRP-2 RDT Cross Reactivity with *Pf*HRP-3 Antigen	3/66	4.5

L 1 2 34 5 6 7 8 9 10 11 12 13 1415 16 17 L

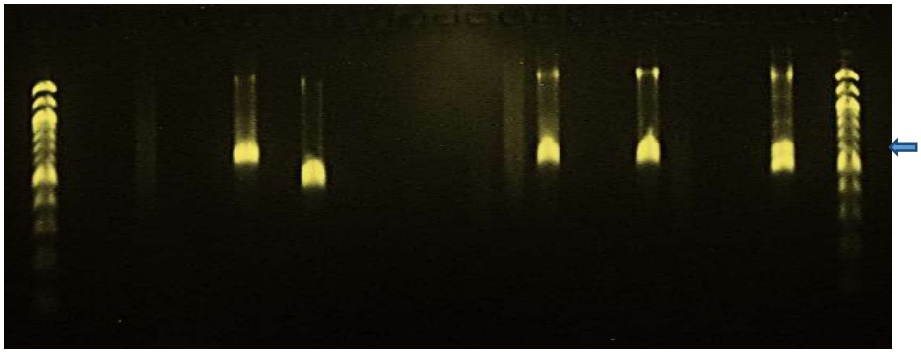


Fig 4.4 *Pf*hrp-2 base pair size

L = 100 bp DNA molecular marker

Well 17 = Positive control

Wells 2 – 16 = *Pf*hrp-2 positive and negativeSamples

Well 1 = Negative control

L 12 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 L

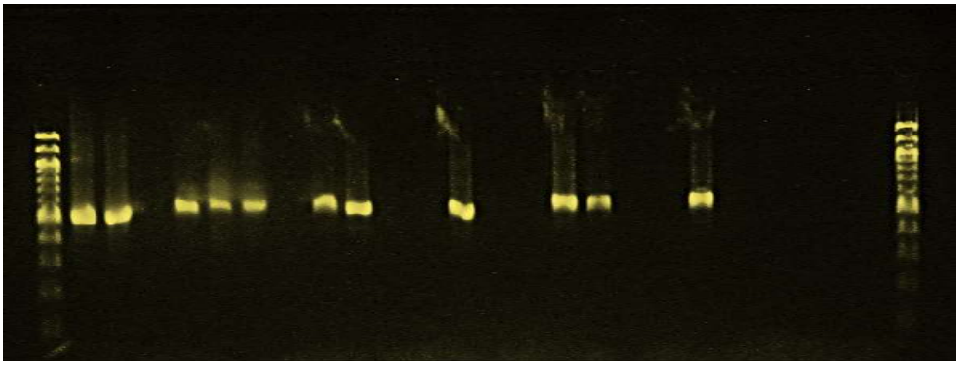


Fig 4.5 *Pf*hrp-3 base pair size

L = 100 bp DNA molecular marker

Wells 18- 21 = Negative controls

Wells 6 – 20 = *Pf*hrp-3 positive and negativeSamples

Wells 1 &17 = Positive controls

4.7 Validation of *P. falciparum* infection with 18srRNA, *msp*1, *msp*2 and *glurp* genes

To further validate that the eleven*Pf*hrp-2 and four*Pf*hrp-3 deleted isolates are*P. falciparum* infected, the 18SrRNA and three polymorphic *P. falciparum* genes, *msp*1, *msp*2 and *glurp* were amplified to confirm the presence of *P. falciparum* infection using specific primers and PCR cycling condition as previously described under methodology. Irrespective of the parasite density quantified microscopically, *Pf*hrp-2 failed to amplify in all eleven isolates (0/11) with duplicate PCR runs, indicating deletion of the *Pf*hrp-2 gene. The*Pf*hrp-3 gene amplified in 63.6% (7/11) while the 18SrRNA gene amplified in all isolates 100% (11/11). The block 2 and 3 regions of *msp*1 and *msp*2 amplified in 72.7% (8/11) respectively while *glurp* R2 region amplified in 81.8% (9/11) (Table 4.7).

Table 4.7. PCR Amplification of Different Genes from RDT False Negative Isolate

RDTStatus	**PD/µL(n=11)**	***Pf*hrp-2**	***Pf*hrp-3**	**18SrRNA**	Msp1	Msp2	Glurp
+	7,330	-	+	+	+	-	+
-	0	-	+	+	+	+	+
-	514	-	-	+	+	+	+
+	1,096	-	+	+	-	+	+
-	0	-	-	+	-	+	+
-	101	-	-	+	+	-	-
-	1607	-	+	+	+	-	-
-	0	-	-	+	+	+	+
-	600	-	+	+	+	+	+
+	38214	-	+	+	-	+	+
-	0	-	+	+	+	+	+
PCR Detection Rate (%)		**0**	**63.6**	**100**	**72.7**	**72.7**	**81.8**

+ = Positive, -- = Negative, PD = Parasite Density

4.8 Genotyping of *msp*2 and *glurp*genes to distinguish recrudescence from re-infection

Among the 289 RDT positive children treated with ASAQ at enrolment, 26 (9.0%) had parasite recurrence by microscopy between days 14 to 28 post treatment. The breakdown showed two enrollees were positive on day 14, sixteen on day 21 and eight on day 28 respectively. There was no case of early treatment failure or early clinical failure among study enrollees. Molecular analysis of paired filter paper samples of day 0 and any other day of parasite recurrence was matched. Allelic genotyping of the*msp2* block 3 (3D7 and FC27) and *Glurp* (RII) regions of the paired isolates were investigated. According to difference in base pair sizes, the results showed that all recurrentparasites were new infections (re-infection) with no evidence of recrudescence because none of the base pair sizes of the allelic families were identical with the day 0 matched isolates. Of the 26 recurrent infections, 22 (84.6%) were from the rural site while 4 (15.4%) were from the urban site (Table 4.11).

Table 4.8 Allelic Genotyping to Distinguish Recrudescence from New Infection

	Day 0 Isolates			Follow up Isolates			
Re-infection Day	**3D7 (bp)**	**FC27 (bp)**	**Glurp (bp)**	**3D7 (bp)**	**FC27 (bp)**	**Glurp (bp)**	**Rmk**
D14 (N = 2)	400	200;800	-	-	-	1200	NI
	500	-	200;800	500	-	1200	NI
D21 (N = 16)	300; 500	-	950	-	400	-	NI
	300,600	-	-	500	-	-	NI
	500	200	-	-	200	-	NI
	500	-	200;800	-	-	1200	NI
	300;400	600	-	-	200	900	NI
	300	-	800	-	200	-	NI
	200;400;700	200	1100	400;600	1100	1200	NI
	200;500	200;300;600	-	500	-	-	NI
	400	400	1100	-	500	1000	NI
	300	0	900	400	400	700	NI
	500	300	-	400	300, 700	-	NI
	600	300;500	-	-	-	900	NI
	400	500	-	-	200	1000	NI
	400	500	-	500	500	1150	NI
	300	500			500	1000	NI
	300; 500		700	400	200	800	NI
D28 (N = 8)	200	600,200	-	500	-	-	NI
	400,900	200;600	-	-	400	-	NI
	200;500	-	-	300;500;800	200;400;500;700	1000	NI
	400,900	400	-	500	-	-	NI
	-	500	1000	-	-	800	NI
	400	300	-	-	200	800	NI
	400	500	-	300; 700	400	1000	NI
	300	500	1000	1000	400, 500, 1000	-	NI

NI = New Infection, Dash = Non amplification of specified marker

4.9 Drug efficacy and treatment outcome

Two children required referral to a tertiary care hospital on account of severe anaemia. The day28 adequateclinicalandparasitologicalcurerate(ACPR) for ASAQ among enrolled children was 78.0% (142/182). There was no case of early treatment failure. After PCR correction (26/40 cases were re-infection), the ACPR of the per protocol population was 94.5%.

Eighty-four (16.4%) children made at least one unscheduledvisitduringthe28-dayfollowup period because ofcaregiver'sworry about delay in resolution of symptoms. A higher proportion of children from the MRDT positivegroupmade unscheduledvisitsthan from the MRDT negative group (23.2% vs 12.0%; $\rho = 0.001$).

4.10 Allelic frequency and multiplicity of infection of *msps* and *glurp* genes

All the day 0 dried blood spots (DBS) filter papers from 511 participants at enrolment, irrespective of lost to follow up or withdrawal of consent was analyzed for *P. falciparum* genetic diversity and prevalence of allelic frequency distribution using the block 2 region of *msp*1(KI, MAD20 and RO33), the block 3 region of*msp*2 (3D7 and FC27)and*glurp*RII region. PCR confirmed 60.5% (309/511) malaria infection. Out of the 309 malaria cases,*P. falciparum* accounted for 250 (80.9%)infections from the rural site and 59 (19.1%) from the urban site. Further genotyping of the allelic frequency distribution and multiplicity of infection (MOI) was evaluated.

Five distinct *msp-1* allelic families were observed. The RO33 allelic family was predominant in the rural site having a frequency of 7.3% monoclonality and fragment size range from 150 – 250bp. The monoclonal infection rate for MAD20 was 3.3% with fragment size range from 150 – 200bp while K1 monoclonal infection was 2.6% with fragment size range from 150 – 350bp respectively. The prevalence of multiclonal infection rate (RO33 + MAD20 + K1) was 82.1% with 2.8% mean multiplicity of infection (MOI) in the rural site. The frequency of monoclonal infection for RO33 and MAD20 and K1 + RO33 was 10.0% respectively in the urban site (Eleta) while the frequency distribution of multiclonal infection (RO33 + MAD20 + K1) was 70.0% with 2.6% multiplicity of infection (MOI) (Table 4.12 and Figure 4.4).

Table 4.9. Distribution of MSP-1 Allelic Frequency in Rural and Urban Sites

Site	*MSP1* family	Amplified samples (N)	Allelic size(bp)	Freq. (%)	MOI
Rural(n = 151)	RO33	11	150 - 250	7.3	
	MAD20	5	150 - 200	3.3	
	K1	4	150 - 350	2.6	
	RO33 + MAD20	4		2.6	
	RO33 + K1	3		2.0	
	RO33 + MAD20 + K1	124		82.1	2.8
Urban(n = 30)	RO33	3	150	10.0	
	MAD20	3	150 - 250	10.0	
	RO33 + K1	3		10.0	
	RO33 + MAD20 + K1	21		70.0	2.6

For the *msp2*, 15 distinct alleles families were detected, eight (8)in 3D7 allelic family with fragment sizes ranging from 200–900 bp and seven (7) in FC27 (200–800 bp) respectively (Table 4.10). The 3D7 allelic family was predominant in the rural site with 43.2% monoclonality of infection, while FC27 monoclonal infection was not detected at the rural site. A high proportionof the samples were multiclonally infected in the rural site with a frequency of 68.3%. In the urban site, both 3D7 and FC27 alleles had 15% frequencies of monoclonal infection while the frequency of multiclonal infection (3D7 + FC27) was 70.0%. The mean MOI for*msp-2* was 2.1 and 1.9 for rural and urban sites respectively (Table 4.10).

Table 4.10. Distribution of MSP-2 Allelic Family in Rural and Urban Sites

Site	*MSP2* family	No. of amplified samples	Allelic size (bp)	Freq. (%)	MOI
Rural (n = 88)	3D7	38	200 – 900	43.20	
	FC27	Nil		0	
	3D7 + FC27	60		68.30	2.1
Urban (n = 20)	3D7	3	300 – 600	15.00	
	FC27	3	400 – 800	15.00	
	3D7 + FC27	14		70.00	1.9

Eleven different *glurp* alleles were distinctly detected in the rural site with base pair sizes ranging from 500 to 1100 bp in a frame of 50 bp bin, coded as genotype I–XII. The predominant allelic family for both rural and urban sites was XI (41.5% and 38.5%). However, only six genotypes (II, VII, IX, X, XI, XII) were detected in the urban site while genotype VI (750–800 bp) was not detected in both rural and urban sites. The mean MOI for both sites was 1.0 (Table 4.11)

Table 4.11. Distribution of Allelic Variants of GLURP RII Repeat Region of *P. falciparum* in Rural and Urban Sites

Genotype	Allelic size (bp)	Rural n = 106 (%)	Urban n = 26 (%)
I	500 – 550	1 (0.9%)	0 (0.0%)
II	550 – 600	2 (1.9%)	1 (3.8%)
III	600 – 650	2 (1.9%)	0 (0.0%)
IV	650 – 700	1 (0.9%)	0 (0.0%)
V	700 – 750	2 (1.9%)	0 (0/0%)
VI	750 – 800	0 (0.0%)	0 (0.0%)
VII	800 – 850	15 (12.2%)	2 (7.7%)
VIII	850 900	6 (5.7%)	0 (0.0%)
IX	950 - 1000	17 (16.0%)	5 (19.2%)
X	1000 - 1050	7 (6.6%)	3 (11.5%)
XI	1050 - 1100	44 (41.5%)	10 (38.5%)
XII		3 (2.8%)	3 (11.5%)
X + XI		2 (1.9%)	0 (0.0%)
XI + XII		4 (3.8%)	1 (3.8%)
III + XI		0 (0.0%)	1 (3.8%)
MOI		**1.0**	**1.0**

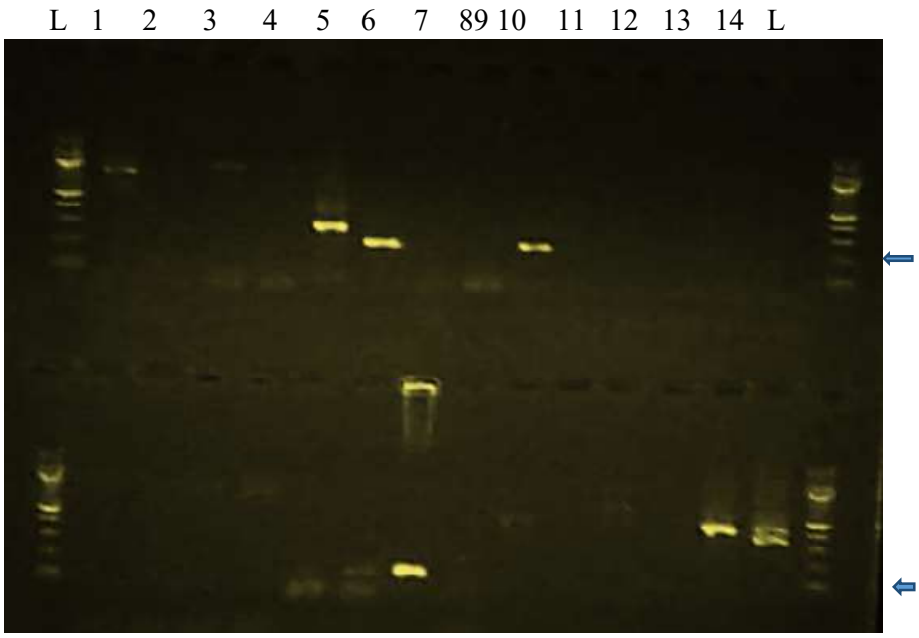


Fig 4.6. Base pair sizes of *msp1*, *msp-2* and *glurp*markers

Down Lane:L = 100bp DNA molecular marker, 14 = *msp2* (3D7) multiclonal infection

13 = *msp2* Positive control, 3 – 12*msp2*= Samples&1 = Negative control

Up Lane: L = 100bp DNA molecular marker, 1 = Negative control*msp2* (FC27), 10 = Positive control, 2 – 9 = Samples, 4 = *glurp* Negative control, 2 & 3 = *glurp* samples, 1 = *glurp* Positive control.

4.11 Allelic frequency distribution of *msp1*, *2* and *glurp* versus parasitaemia

Parasite density was categorized into ≥ 2000 and ≤ 2000 parasites/µL tocomparethe extent of allelic frequency distribution of *msp1*, *msp2* and *glurp* genes. At parasitaemia ≥ 2000 parasite/µL,theRO33, MAD20 and K1 allelic families were monoclonally detected in 11.0% (9/82), 4.9% (4/82) and 3.7% (3/82) respectively. While the prevalence of multiclonal infections for *msp*1was 74.4% (61/82) and 2.6% MOI.

Similarly, FC27 was positive for 18.6% (8/43) while 3D7 accounted for 34.9% (15/43). The *msp2* multiclonal infection was 46.5 % (20/43) and 1.8% MOI. A slight decrease in the prevalence of allelic frequencies was observed at parasite density ≤ 2000 parasites/µL. However, the difference was not statistically significant as K1, MAD20 and RO33 had 1.0% (1/99), 4.0% (4/99) and 5.1% (5/99) monoclonal infections while multiclonality of infections was 84.8% (84/99) and 2.8% MOI respectively. For *msp-2*, 3D7 amplified for 13.0% (12/92) while FC27 recorded 28.3% (26/92) monoclonal infection while multiclonal infection at parasite was 58.7% (54/92) and 2.0% MOI respectively.

At ≥ 2000 parasites/µL, the allelic frequency for the RII region of *glurp* gene was 97.0% (64/66) while 100% (66/66) was recorded at parasite density ≤ 2000 parasites/µL. The MOI for both categories was 1.1 and 1.0 respectively (Table 4.12). An increased multiplicity of infection at parasite density ≤ 2000µL was observed but not statistically significant ($p > 0.05$).

Table 4.12. Distribution of MSP-1, MSP-2 and GLURP Alleles Frequency by Parasite Density among Children

Allelic Family	Parasite count>2000/ µL	Allelic size (bp)	Parasite count<2000/µL	Allelic size (bp)	MOI
MSP1	**n = 82 (%)**		**n = 99 (%)**		
RO33	9(11.0)	150 – 250	5(5.1)	150 - 250	
MAD20	4(4.9)	150 – 200	4(4.0)	150 - 300	
K1	3(3.7)	150 – 300	1(1.0)	350	
RO33 + MAD20	1(1.2)		2(2.0)		
RO33 + K1	3(3.7)		3(3.0)		
MAD20 + K1	1(1.2)		0(0.0)		
RO33+MAD20+K1	61(74.4)		84(84.8)		2.8
MSP2	**n = 43 (%)**		**N= 92(%)**		
3D7	15(34.9)	300 – 900	12(13.0)	200 - 600	
FC27	8(18.6)	200 – 600	26(28.3)	300 - 700	
3D7 + FC27	20(46.5)		54(58.7)		2.0
GLURP	**n = 66(%)**		**n = 66(%)**		
RII	64(97.0)	500 - 1050	66(100.0)	500 - 1050	1.0

4.12 Amino acid nucleotide repeat types in *Pf*hrp-2/*Pf*hrp-3 conserved isolates

A total of ten *Pf*hrp-2 and twelve *Pf*hrp-3 sequences obtained from eleven RDT positive and one RDT negative samples were translated successfully into amino acids. For *Pf*hrp-2, a total of twelve previously described *Pf*hrp-2 amino acid repeat types were identified. The *Pf*hrp-2 repetitive sequence began with the repeat type 1 (AHHAHHVAD) and ended with repeat type 12 (AHHAAAHHEAATH) motif. Repeat types 9, 11 and 19 did not occur. The structural organization of the *Pf*hrp-2 amino acid repeat types was highly diverse. On the other hand, eight out of nine previously described *Pf*hrp-3 repeat types were identified. Types 1, 4 and 7 motifs were present in both *Pf*hrp-2 and *Pf*hrp-3 amino acid tandem repeats. There was 100% occurrence of types 4 and 7 repeats that were believed to be the epitope that codes for RDT target antigen. Repeats types 15 (AHHAHHAAN), 16 (AHHAAN), 17(AHHDG), 18 (AHHDD) and 20 (SHHDD) were distinctly detected in *Pf*hrp-3 while types 3, 5, 6, 8, 10 12, 13 and 14 were absent in *Pf*hrp-3. The types and total number of repeats, and the number of individual repeats within *Pf*hrp-2 and *Pf*hrp-3 in successfully sequenced isolates are shown in Table 4.12. No significant difference was observed in the types of repeats in individual isolates.

Table 4.13. Amino acid nucleotide Repeats Observed in *Pf*hrp-2 and *Pf*hrp-3

		Antigens observed	
Repeat Type	**Repeat sequences**	***PF*HRP-2** **n = 10 (%)**	***PF*HRP-3** **n =12 (%)**
1	AHHAHHVAD	10 (100)	12 (100)
2	AHHAHHAAD	10 (100)	0
3	AHHAHHAAY	9 (90)	0
4	AHH	10 (100)	12 (100)
5	AHHAHHASD	9 (90)	0
6	AHHATD	10 (100)	0
7	AHHAAD	10 (100)	12 (100)
8	AHHAAY	9 (90)	0
9	AAY	0	0
10	AHHAAAHHATD	10 (100)	0
11	AHN	0	0
12	AHHAAHHEAATH	10 (100)	0
13	AHHASD	1 (10)	0
14	AHHAHHATD	2 (20)	0
15	AHHAHHAAN	0	12 (100)
16	AHHAAN	0	12 (100)
17	SHHDG	0	2 (17)
18	AHHDD	0	12 (100)
19	AHHAA	0	0
20	SHHDD	0	12 (100)

CHAPTER FIVE

5.0 Discussion

The deployment and utilization of *Pf*HRP-2 based RDTs has tremendously increased in the last decade which has enhanced malaria diagnosis and case management across all health facilities and particularly in resource limited settings(Djallé *et al*., 2014). However, the occurrence of *Pf*hrp-2 gene deletions in parasite population across geographically endemic areas is a major challenge to accurately diagnose malaria with the*Pf*HRP-2 based RDTs. In addition, the genetic variability of infecting parasite strains plays a major role in the parasite dynamics and it is imperatively essential to continuously evaluate the parasite genetic structure in order to provide timely and appropriate intervention programmes toward effective malaria containment and avert the occurrence of parasite resistance to currently available antimalarial drugs. This study reported the performance of a*Pf*HRP-2 based RDT, RDT false negative isolates for Pfhrp2/Pfhrp-3 deletion, thenucleotide variability among *Pf*hrp-2 and *Pf*hrp-3 conserved isolates and the prevalence of allelic frequency distribution and genetic diversity of*P. falciparummsp1*, *msp*2 and *glurp*genes in parasite sub- population in Ibadan, southwest Nigeria.

Baseline clinical and demographic findings in the two centers were similar. However, malaria positivity in the rural site was significantly higher compared to the urban site ($p < 0.001$). Fever with axillary temperature $\geq 37.5\ ^{o}$ C was observed in most of the enrollees with significantdecrease in hematocrit level for malaria positive enrollees. The decreased hematocrit level was not surprising because *P. falciparum* malaria infects all stages of red blood cells (Mohandas & An, 2012). Malaria infection is a notable cause of anaemia, resulting from the destruction of parasitized RBCs, the rapid destruction of normal RBCs and dyserythropoiesis (Lee *et al.,* 2008). A large proportion of malaria infections detected in this study was caused by *P. falciparum* mono-infections, the most virulent *Plasmodium*specie and most common in the study locations. Mixed infection with either *P. malaria* or *P. ovale* was also detected but no *P. vivax* was identified during PCR speciation. The absence of *P. vivax* in the study population maybe as a result of the absence of the Duffy antigen receptor for chemokines (DARC). It has been reported that about 95 – 99% of individuals in tropical Africa are mostly negative for the Duffy antigen that mediates the invasion of reticulocytes by *Plasmodium vivax* (Howes *et al.,* 2015; Liu

et al., 2014).Although, there are recent reports of *P. vivax* infection in Duffy negative individuals in Republic of Benin, West Africa (Poirier *et al.,* 2016).

5.1 Evaluation of *Pf*HRP-2 based RDT performance

The study evaluated the performance of *Pf*HRP-2 based RDTs with the aim of recommending total compliance to the parasite-based malaria case management using RDTs in areas where basic microscopy is not available. Malaria rapid diagnostic tests are vital for early diagnosis and accurate treatment of confirmed cases which improve the standard of care in malaria endemic areas. The evaluated malaria RDT (SD-Bioline™), was optimally sensitive (95 - 97%) and a specificity of 67 - 73% when compared with microscopy.Several studies has been conducted to evaluate RDT performance across malaria endemic regions to validate RDTs usefulness in providing effective and definitive treatment to reducing malaria burden with varying level of sensitivity and specificity(Jimenez *et al*., 2017). The low specificity recorded by RDT in this study and elsewhere is not surprising in areas of high malaria transmission due to the occurrence of false positive results(Tiono *et al*., 2013; Abeku *et al.,* 2008; Hopkins *et al.,* 2013). Which could be caused by the persistence of *Pf*HRP-2 antigenemia following treatment of *falciparum* infection(Chaijaroenkul *et al.,* 2011; Falade *et al.,* 2016; Gitonga *et al.,* 2012). It has been reported that the *Pf*HRP-2 antigen and uncleared gametocytes circulates in blood to continuously express *Pf*HRP-2 antigen from previously infected but treated individuals and persist between 2 – 4 weeks after asexual parsite clearance(Iqbal *et al.,* 2004; Mayxay *et al.,* 2001). In this study, about 31% RDT false positive cases were detected when microscopy was considered the gold standard. PCR-corrected RDT reading recorded 27.3% RDT false positive results while PCR-corrected microscopy reading accounted for only 9.0% microscopy false positive results. The prevalence of RDT false positive results is therefore an indication for highmalaria transmission in endemic regions which requires additional malaria control efforts(Murungi *et al*., 2017). The implication of RDTs false positive results is that patients without parasitaemia will be wrongly administeredantimalarial drugs. The study drug (artesunate/amodiaquine) was given to RDT false positive participants since treatment was based on RDT outcome, thereby, exposing them to unnecessary antimalarial medication and possible adverse drug reactions. However, the proportion of false positive results are relatively insufficient to affect the programmatic deployment of RDTs as viable option for malaria diagnosis in the study area.Despite the low specificity, sensitivity was high,which implies that RDTs can be utilized as an alternative to microscopy in southwest Nigeria and in clinical settings to

augment microscopy in line with the parasite based treatment of suspected malaria cases as recommended.

Another major factor that has threatened RDT appllicability across malaria endemic regions is the occurrence of false negative readings(Berhane *et al*., 2018). Rarely, operator errors during performance and interpretation of RDT results can be reported as false-negative (WHO, 2016). False negative RDT results has also been linked to asymptomatic malaria infection in high transmission areas. A study conducted in Pakistan reported that RDT failed to pick any positive malaria infection among asymptomatic individuals (Naeem *et al.,* 2018). But more worrisomely, is the reported deletion of *Pf*hrp-2 and *Pf*hrp-3 genes in parasite population (Abdallah *et al.,* 2015; Akinyi *et al.,* 2013) which causes a greater rate of false negative results. A large proportion of *Pf*hrp-2/3 gene deletion was first reported in the Peruvian Amazon and it gradual spread across geographical locations of malaria endemic areas (Gamboa *et al.,* 2010). Similar studies conducted in Africa also confirmed *Pf*hrp-2 gene deletions in Ghana, Mali, Rwanda and Senegal (Amoah *et al.,* 2016; Koita *et al.,* 2012; Kozycki *et al.,* 2017; Wurtz *et al.,* 2013).

This study recorded *Pf*hrp-2 and *Pf*hrp-3 deletion among parasites in Nigeria. Unlike the study reported by Gamboa *et al*, where about 70% of the evaluated isolates lacked *Pf*hrp-3 and 41% *Pf*hrp-2 genes, our study recorded a lower proportion of gene deletion (16.7% *Pf*hrp-2 and 6.0% *Pf*hrp-3). This was higher than 2.4% that was reported in Senegal (Wurtz *et al.,* 2013). Findings from Mali reported 45.5%, Ghana 33.3% and Rwanda has 23% deletion of *Pf*hrp-2 gene. Recent findings from Eritrea has the highest proportion of *Pf*hrp-2 and *Pf*hrp-3 gene deletion in the world accounting for over 80% gene deletion (Berhane *et al.,* 2018). The report suggested that malaria endemic regions are at risk of *Pf*hrp-2 and *Pf*hrp-3 gene deletion, thereby recommending nation-wide mapping of the prevalence of *Pf*hrp-2 and *Pf*hrp-3 deleted parasites to inform malaria case management policies. The study concluded that *Pf*HRP2-based RDTs are inappropriate for malaria diagnosis in Eritrea.

The cause of parasites lacking *Pf*hrp-2 genes is not well understood. Although, some reports have associated it with genetic diversity of amino acid repeats sequence (Deme *et al.,* 2014; Kumar *et al.,* 2017),while Koita *et al* associated it to asymptomatic malaria. More work is however, required to elucidate the parasite dynamics in providing a definitive cause of parasites without the *Pf*hrp-2 gene.

The role *Pf*hrp-3 gene has not been well characterized, but evolutionary studies linked both genes as having the same ancestral origin with identical amino acid sequence(Wellems & Howard, 1986; Wellems *et al.,* 1987). The *Pf*hrp-3 gene has been reported to cross react with *Pf*HRP-2 based RDTs (Bharti *et al.,* 2017). Report in this study, recorded three cases (4.5%) of *PfH*RP-3 antigen cross reactivity with *Pf*HRP-2 based RDTs to give a positive RDT results. A study from Kenya reported a similar proportion of *Pf*hrp2/3 deletions with a more conserved *Pf*hrp-3 gene that cross reacted with HRP-2 based RDT to give a positive test result (Beshir *et al.,* 2017). It is likely that in sub Saharan Africa, there may be minimal level of *Pf*hrp-2 deletion across the continent with a more intact *Pf*hrp-3 that may cross react with *Pf*hrp-2 based RDTs at a high parasite density.

5.2 Efficacy of artesunate/amodiaquine treatment

Artesunate/amodiaquine (ASAQ) is one of the first line ACTs used for malaria chemotherapy in Nigeria. The efficacy of ASAQ as the study intervention drug was affirmed and was in consistence with previous report by Falade et.al on the high efficacy of ASAQ in malaria treatment (Falade *et al.,* 2008). None of the malaria positive participants treated with ASAQ developed early treatment failure and there was no confirmed cases of danger signs of malaria or severe malaria on days 1 and 2 post treatment or asexual parasites detected on day 3. The study did not find parasitaemia on day three ≥25% of the initial parasitaemia on days 0 and 3 with an axillary temperature ≥37.5°C. Although, twenty-six recurrent infections were observed during the 28-day follow up. Two enrollees had recurrent infections on day 14, sixteen on day 21 and eight on day 28 post treatment. Allelic genotyping of the *P. falciparum* msp1, msp2 and glurp genes as recommended by WHO to discriminate recrudescence infections from new infections indicated all the recurrent infections were new infections. This may probably reflect the high malaria transmission in the study areas. About 84.6% (22/26) of the re-infected cases was from the rural site, indicating that malaria transmission and it's burden is still more intense in rural settings than urban as confirmed in this report and in previous studies (Govoetchan *et al.,* 2014).

5.3Frequency distribution and allelic diversity of *msp1*, *msp2* and *glurp* genes

The genetic characterization and mapping of *P. falciparum* antigenic biomarkers (msp1, msp2 and glurp) are essential molecular techniques in understanding the parasitedynamics in malaria endemic regions with varying transmission intensity across different geographical areas and to study the population structureof the parasites to develop effective control strategies (Jennison *et al.,* 2015; Razak *et al.,* 2016). The complex genetic structure and diversity of *P. falciparum* is a major setback in developing vaccines targeting the merozoite surface proteins and glutamate-rich (Ouattara *et al.,* 2015). Findings in this study and elsewhere showed a high frequency of allelic diversity among parasite strains in the study region (Duru & Thomas, 2014; Happi *et al.,* 2004). There was similar pattern of high diversity betweenthe rural and urban sites with, RO33 of the *msp*1 gene as the most frequentallele genotype for both rural and urban sites.This wascontrary to earlier findings that reported the K1 allele genotype as the most predominant allelic family in thesame region (Happi *et al.,* 2004; Olasehinde *et al.,* 2012). Ironically, findings in the study showed K1 genotype was less frequentin both sites. Although, all the studieswere conducted in the same region, however, the patients population in the Happi *et al* study was predominated with oneethnic group with relatively small sample size of fifty and many years ago since the study was conducted (14 years) maybe responsible for the allelic genotype difference as found in the present study which can be correlated with the uniqueness and complex structure of the parasite dynamics over time. Furthermore, thehighrate of MOI in this study confirmed the complex genetic structure and multiple strainsof the infecting parasites in the population. Factors that may contribute to the diverse genetic make-up of theparasite populationmay be high intensity of transmission in the study area and parasite selection pressure from different interventional measures such as chemotherapy, the use of indoors residual sprays (IRS)and insecticide-treated nets (ITNs). Alteration in thegenetic make-up of *P. falciparum*has been reported in endemic and high transmission settings following change in national anti-malarial drugs policies and introduction of ITNs (Gatei *et al.,* 2015) . Similarly, the finding of RO33 as the most predominant allelic genotype was also reported in Senegal (Robert *et al.,* 1996), Sudan (Hamid *et al.,* 2013), Uganda (Peyerl-Hoffmann *et al.,* 2001), and Thai-Myanmar borders (Congpuong *et al.,* 2014b), which were correlated totransmission intensity and reduced response to artesunate-mefloquine therapy. On the

contrary, reduced efficacy or response to ASAQ was not recorded despite the high prevalence of RO33 allelic genotype in this study.

The 3D7 allelic genotype was themost predominant allele of the *msp*2 locus for both sites. Similar findings from the Thai-Myanmar border(Congpuong *et al.,* 2014b), Nigeria (Amodu *et al.,* 2008) Congo Brazzaville (Mwingira *et al.,* 2011), as well as Peru and Iran (Chenet *et al.,* 2008b; Zakeri *et al.,* 2005) reported the preponderance of 3D7 allelic genotype in the parasite population. However, this was contrary to studies from Osogbo, Nigeria (Ojurongbe *et al.,* 2011) and northeastern Myanmar (Yuan *et al.,* 2013). Variation in the *msp*2 alellic frequency and distribution has been associated with the presenting clinical pattern of infected individuals with conflicting reports. Some studies associated the high frequency of RO33 and FC27 alleleswith asymptomatic malarialinfections (Aubouy *et al.,* 2003), while others have reported this not to be (Amodu *et al.,* 2008; Kyes *et al.,* 1997; Ntoumi *et al.*).

Multiclonal infection observedin *msp*1 and *msp*2 genotypes in both sites were relatively high indicating the presence of multiple parasite strains in the region. The existence of a high geneticdiversity demonstrated a large parasite population size that can accommodate different parasites for effective hybridization and recombination of genotypes. Human migrationcan also transfer different parasite strains across geographical boarders thereby increasing the risk of genetic diversity among parasites(Mita & Jombart, 2015). The data in this study showed a significant *msp*1 variants of about 350 bp allelic sizes with similar*msp*2 sizes which was consistence with previous report (Happi *et al.,* 2004), contributingto the parasite dynamics and diversity in southwest Nigeria. It is worthy of note that the highest allelic band size of350 bp was more predominantin the rural setting, an indication that parasite species in rural settings may have higher rate of genetic recombination thanurban areas.

The *glurp* RII repetitive regionalso demonstrated a high genetic diversitywith12 different allelic fragment sizes genotyped. Eleven of these fragment sizes was observed in the rural setting while six was observed in theurban site. The high frequency and distribution of *glurp* allelic genotype inthe study sites are not surprising, considering the fact that malaria is endemic with a hightransmission intensity which has also been previouslyreported (Duru & Thomas, 2014; Kumar *et al.,* 2014). The lower rate of *glurp* allelic genotypeobserved in the urban site in contrast to the ruralarea may be also

correlated to higher malaria transmissionintensity in the rural site with significant parasite genetic diversity evolving at a faster rate in therural than urban areas.

When parasite density was categorizedinto two, samples with parasitaemiagreater than 2000 parasites/µL and less than2000 parasites/µL.Analysis of these two categories, especially parasite densities at over 2000 parasites/µL did not influence genetic diversity in the parasite population. Interestingly, at less than 2000 parasites/µL, there was a higher multiplicity of infection and genetic diversity but the difference was not statistically significant, indicating that high parasitaemia may have a weaker associationwith MOI and genetic diversity in the parasite population in the study area.The correlation between *P. falciparum* genetic diversity and severity ofmalaria infection (indicated as high parasitaemia in this study)has been generating conflicting reports from different research findings. A study conducted by Ojurongbe *et., al* did not establish any significant correlation between differentallelic genotypes of *msp*2 (3D7 & FC27) and parasite density (Ojurongbe *et al.,* 2011). However, the reverse was the case as strong association between parasite density and allelic diversity on the frequency of *msp*1 and *msp*2 allelic genotypes (K1, RO33, MAD20 and 3D7, FC27) were reported from studies conducted in Central Sudan (Hamid *et al.,* 2013) and CongoBrazzaville, (Mayengue *et al.,* 2011).Additionally, a previous study from Ibadan reported an association between the *msp*2 locus and clinical severity of malaria in children (Amodu *et al.,* 2008), but this finding was contrary and was not reproduced in this study. Although, this study enrolled children who had fever in the preceding 48 hours, which may have given opportunity to parents/guardians to administer antipyretic drugs prior to presentation for enrollment. However, it is not impossible for a positive correlation between severity of malaria infection and allelic diversity to exist in malaria hyper-endemic regions because of some associated factors, including co-infections with multiple parasite strains or, super-infections, intra-host dynamics such as the overlapping of different parasite strains between primary and secondary infections, and host immunity andexposure variations are some of the contributory factors previously reported (Conway, 2007; Pacheco *et al.,* 2016; de Roode *et al.,* 2005).

5.4 Analysisof amino acid nucleotide repeats sequences

*Pf*HRP-2 based RDTs have demonstrated optimal sensitivity in malaria diagnosis. However, genetic diversity, nucleotide variability and *Pf*hrp-2 gene deletion has been reported to affect RDT sensitivity (Kumar *et al.,* 2014). In this study, 12 of 20 previously reported amino acid tandem repeats sequence around the globe were detected as

previously described(Baker *et al.,* 2005, 2010; Deme *et al.,* 2014) for *Pf*hrp-2 and 9 repeats types for *Pf*hrp-3, indicating that isolates from Nigeria are highly variable in their amino acid repeats sequence. An extensive variability in fragment sizes and amino acid repeats was more predominant in *Pf*hrp-2 than *Pf*hrp-3. The effect of sequence variation of antigens to the binding of specific monoclonal antibodies has been reported to affect the sensitivity of *Pf*HRP-2 based RDTs, if the epitopes recognized in the repeat sequence are not universally present among parasite stains (Kumar *et al.,* 2014; Lee *et al.,* 2006). Interestingly, types 2 and 7 repeats, which are the suggested target epitopes recognized by *Pf*HRP-2 specific monoclonal antibodies was detected in all isolates as previously reported (Baker *et al.,* 2005, 2010; Kumar *et al.,* 2014; Mariette *et al.,* 2008). This implies that RDT sensitivity in *P. falciparum* diagnosis will not be significantly affected at parasite densities ≥ 250µL.

Unlike the Deme *et al* findings, were isolates from Senegal, Mali and Uganda were lacking Type 11 and 14 repeats, the Nigeria isolates lacked Type 11 repeats but Type 14 repeats was present, which was similar to isolates from Thailand and Papua New Guinea (Baker *et al.,* 2010). Similarly, Type 9 repeats was absent in Nigeria as it was reported in Mali and Ugandan isolates but was present in Senegalese isolates. The observed variation in the distribution of amino acid repeats types maybe attributable to difference in geographical location, transmission intensity and infecting parasite strains, which could provide an insight to the variability in RDT performance.

Furthermore, findings in this study showed more diversity of *Pf*hrp-2 nucleotide sequence than *Pf*hrp-3. The presence of type 2 and type 7 repeats in the *Pf*hrp-3 sequence was evidenced by the cross-reactivity of *Pf*hrp-3 antigens on isolates lacking *Pf*hrp-2 gene as observed in this study. This has been earlier reported by Wellens *et al.* that about 80% - 90% nucleotide similarities exists between *Pf*hrp-2 and *Pf*hrp-3 genes, that antibodies present in *Pf*HRP-2 based RDTs can cross react with *Pf*HRP-3 antigens.

The *Pfhrp*-2and *Pfhrp*-3gene*s* are found in the sub-telomeric regions of chromosome 8 and 13 of the *Plasmodium falciparum* genome (Rachid Viana *et al.,* 2017). Generally, genes located in the sub-telomeric and telomeric regions of *Plasmodium* are associated with high frequency of genetic diversity and are prone to mutational changes during genetic recombination (Figueiredo *et al.,* 2002). Some of the factors implicated in the generation of amino acid sequence repeats and variation include slipped strand mis-pairing, abnormal or error in DNA replication or repair, unequal reciprocal combination

and gene conversion (O'Dushlaine *et al.,* 2005; Richard *et al.,* 1999). The different organization and varying number of repeats observed in *Pfhrp*-2and *Pfhrp*-3are likely from frequent recombination of chromosomes. Therefore, a correlation between malaria transmission intensity and *Pfhrp*-2 nucleotide variation is not surprising to occur in malaria endemic region like Nigeria, which is often associated with co-infection of multiple strains that can influence genetic recombination during gametocyte reproduction in the definitive host. The different types of *Pfhrp-2* and *Pfhrp-3* amino acid sequence repeat observed in this study is indicative of high malaria transmission intensity in the area.

CHAPTER SIX

6.1 Summary and conclusion

The performance of *Pf*HRP-2 based rapid diagnostic tests (RDTs) are comparable with microscopy and can be deplored for effective and parasitological based malaria diagnosis in rural settings for appropriate malaria treatment. However, low proportion of *P. falciparum* parasites in southwest Nigeria are lacking *Pf*hrp-2 and/or *Pf*hrp-3 genes which may cause RDT false negative results. Though, the reported proportion of deletions did not affect the performance of *Pf*HRP-2 based RDTs in malaria diagnosis in this study, suggesting the continuous utilization of *Pf*HRP-2 based RDTs in settings where microscopy is not available. However, there is need to incorporate parasite lactase dehydrogenase (pLDH) based RDTs for optimal detection of parasites lacking the *Pf*hrp2 gene.

An extensive genetic diversity of *P. falciparum* merozoites surface proteins (*msp*1 &*msp*2) and glutamate-rich protein (*glurp*) existed among *P. falciparum*isolates in Ibadan. There was no statistical significance between parasite density in relation to genetic diversity in rural and urban sites. Although, malaria prevalence was higher in rural than urban area. Additionally, very high rate of nucleotide variability of the various amino acid tandem repeats types were detected in *Pf*hrp-2 and *Pf*hrp-3 sequences. The efficacy arm of the study confirmed high efficacy of artesunate/amodiaquine despite the genetic variability of the infecting parasite strains. No recrudescence parasite was detected, all genotyped and matched isolates were confirmed to be new infections. However, a large scale nation-wide study to map the geographical distribution of *Pf*hrp-2 and *Pf*hrp-3 genes deletion and its evolutionary trend is warranted to guide malaria intervention programmes in Nigeria.

6.2 Contribution to knowledge

- Rapid diagnostic tests can be used in guiding malaria case management, especially in peripheral health facilitieshaving shown a moderate performance when compared with microscopy and PCR.
- *Plasmodium falciparum* in Nigeria has spontaneous *Pf*hrp-2 and*Pf*hrp-3 gene deletion which can cause false negative RDT results.
- Artesunate/amodiaquine combination is still efficacious as no recrudescence parasite was genotyped.
- There is an extensive genetic diversity of merozoite surface proteins and glutamate-rich protein among the parasite strains
- High nucleotide sequence variation exist in the *Pf*hrp-2 and *Pf*hrp-3 genes

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Appendix 1

Description of Primers and Reagents

10X TBE Buffer Stock Solution

The 10X TBE (amresco™) containing Tris base, Boric acid and EDTA was purchased from Inqaba African Biotec Ltd.

Preparation of 1X TBE working solution

To prepare the 1X TBE working solution from the 10X TBE, add 10ml of the 10X TBE buffer into 90ml of distilled water for 100ml of 1X TBE buffer solution.

Preparation of 1.5% Agarose Gel

Measure 100ml of 1X TBE buffer solution and weigh 1.5g agarose powder (CSL-AG100g. Clever Scientific Limited. UK) into a conical flask. Mix thoroughly and heat mixture in a microwave until boiling (3 - 5 min), making sure that all agarose particles are dissolved. Add 2µL ethidium bromide and mix rigorously for 1 min. Cool to 50 °C and pour in gel casting mold. Allow the cast gel to set for 30 min at room temperature and remove the well-forming combs embedded in the electrophoretic mold to create wells for loading of PCR products.

Preparation of 100µM primer stock solution

Briefly centrifuge lyophilized oligo pellets in tube before opening to prevent loss of the pellet. Stock solution of oligos was prepared (100mM = 100 pmole/µL) with PCR nuclease free water at pH 7.5. The concentrated oligos was stored at -20^{O}C. To avoid frequent freeze-thaw cycles, aliquots of the stock solution was divided into smaller volumes for long term storage and to prevent accidental contamination.

Volumes of Stock Dilution for Primers

rPLU5 (Plasmodiun forward primer) add 608.58µL PCR grade water

rPLU6 (Plasmodiun reverse primer) add 413.94µL PCR grade water

rFAL1(*P. falciparum* forward primer) add 393.99µL PCR grade water

rFAL2 (*P. falciparum* reverse primer) add 439.31µL PCR grade water

FwMAL1 (*P. malariae* forward primer) add 527.9µL PCR grade water

RvMAL2 (*P. malariae* reverse primer) add 523.62µL PCR grade water

FwOVA1 (*P. ovale* forward primer) add 446.1µL PCR grade water

RvOVA2 (*P. ovale* reverse primer) add 447.46µL PCR grade water

FwVIV1(*P. vivax* forward primer) add 562.31µL PCR grade water

RvVIV2 (*P. vivax* reverse primer) add 482.17µL PCR grade water

FC 27-1(msp2/FC27 forward primer) add 485.01µL PCR grade water

FC 27-2 (msp2/FC27 reverse primer) add 544.13µL PCR grade water

3D7- 1 add 442.13µL PCR grade water

3D7- 2 add 441.18µL PCR grade water

Msp1F add 574.31µL PCR grade water

Msp1R add 628.34µL PCR grade water

K1F add 455.29µL PCR grade water

K1R add 512.93µL PCR grade water

Ro33F add 453.16µL PCR grade water

Ro33Radd 543.7µL PCR grade water

Mad20Fadd 613.23µL PCR grade water

Mad20Radd 507.16µL PCR grade water

G-F3add 489.76µL PCR grade water

G-F4add 431.08µL PCR grade water

G-NFadd 519.59µL PCR grade water

Reconstitution of Stock Primers to 1X Working Solution for PCR Amplification

To reconstitute stock primer solution to a 1X working solution, a ratio of 10 : 90µL (e.g. add 10µL stock primer solution into 90µL PCR grade water) was prepared to form 1X(100µL) working solution.

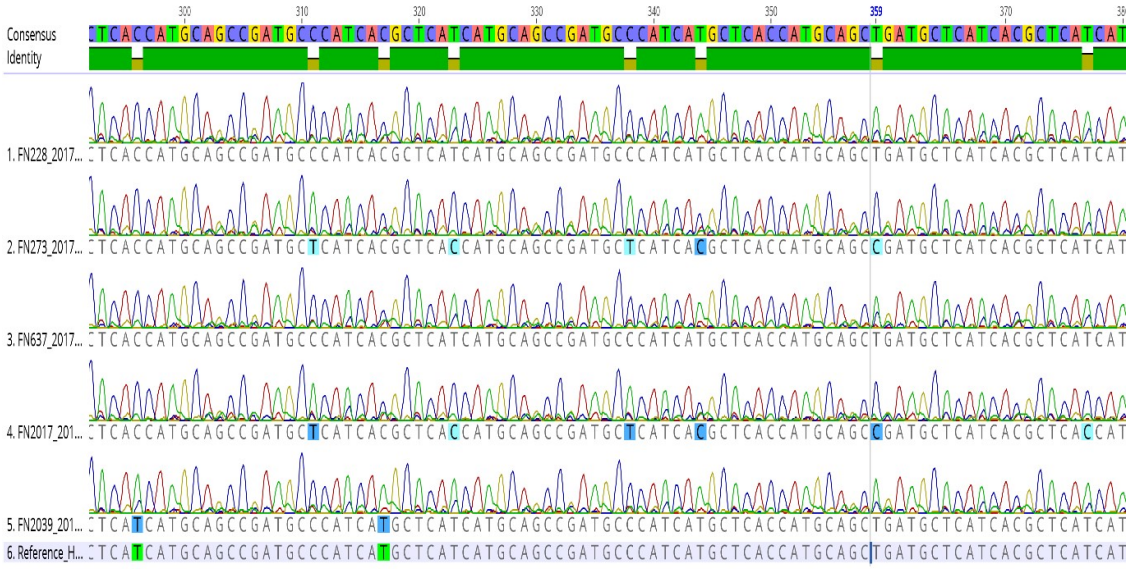
Primer Pairs and Sequences

PRIMER NAME	Sequence (5' to 3')	Basepair Size	Target gene
Plasmodium			
rPLU5F	CCTGTTGTTGCCTTAAACTT		18SrRNA
rPLU6: R	TTAAATTGTTGCAGTTAAAACG		
P. falciparum			
rFAL1 F	TTAAACTGGTTTGGGAAAACCAAATATATT	205	18SrRNA
rFAL2 R	ACACAATGAACTCAATCATGACTACCCGTC		
P. ovale:			
FwOVA1 F	ATCTCTTTTGCTATTTTTTAGTATTGGAGA	800	18SrRNA
RvOVA2 R	GGAAAAGGACACATTAATTGTATCCTAATG		
P. malariae:			
RwMAL1 F	ATAACATAGTTGTACGTTAAGAATAACCGC	145	18SrRNA
Rv MAL2 R	AAAATTCCCATGCATAAAAAATTATACAAA		
P. vivax		117	
FwVIV 1 F	CGCTTCTAGCTTAATCCACATAACTGATAC		18SrRNA
RvVIV 2 R	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA		
MSA 2-1: F	ATGAAGGTAATTAAAACATTGTCTATTATA		
MSA2-4: R	TTATATGAATATGGCAAAAGATAAAACAAG		MSP2 Gene
FC 27-1: F	GCAAATGAAGGTTCTAATACTAATAG	200 - 600	
FC27-2: R	GCTTTGGGTCCTTCTTCAGTTGATTC		FC27
3D7-1: F	GCAGAAAGTAAGCCTTCTACTGGTGCT	200 - 800	
3D7-2: R	GATTTGTTTCGGCATTATTATGA		3D7
G-F3: F	ACATGCAAGTGTTGATCCTGAAG		GLURP
G-F4: R	TGTAGGTACCACGGGTTCTTGTGG		Glurp
G-NF: F	TGTTCACACTGAACAATTAGATTTAGATCA	500 - 1200	R2
G-F4: R	TGTAGGTACCACGGGTTCTTGTGG		
MSP1 F	AAGCCTTAGAAGATGCAGTATTGAC		Msp1
MSP1 R	ATTCATTAATTTCTTCATATCCATTATC		
K1 F	AAGAAATTACTACAAAAGGTG	200 - 600	

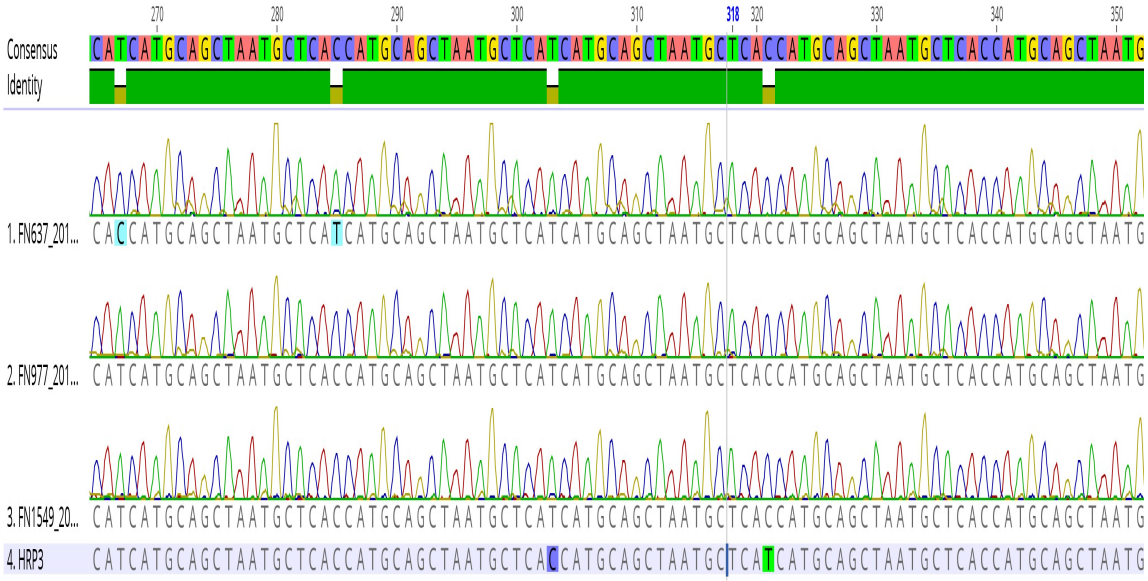
K1 R	TGCATCAGCTGGAGGGCTTGCACCAC		
RO33 F RO33 R	AGGATTTGCAGCACCTGGAGATCT GAGCAAATACTCAAGTTGTTGCA	200 – 600	RO33
MAD20 F MAD20 R	TGAATTATCTGAAGGATTTGTACGTC GAACAAGTCGAACAGCTGTTA	200 – 600	MAD20
Pfhrp2 – F1 Pfhrp2 – R1	CAAAAGGACTTAATTTAAATAAGAG AATAAATTTAATGGCGTAGGCA	600 – 900	hrp2
Pfhrp2 – F1 Pfhrp2 – R1	ATTATTACACGAAACTCAAGCAC AATAAATTTAATGGCGTAGGCA	500 – 900	exon 1
Pfhrp3 – F1 Pfhrp3 – R1	AATGCAAAAGGACTTAATTCTGGTGTAAGT GATGCGTAGT		hrp3
Pfhrp3 – F2 Pfhrp3 – R1	AAATAAGAGATTATTACACGAAAG TGGTGTAAGTGATGCGTAGT	500 – 900	exon 1

Appendix 2

*Pf*hrp-2 and *Pf*hrp-3 Nucleotide Sequence Alignment



*Pf*hrp-2 Nucleotide Sequence Alignment



*Pf*hrp-3 Nucleotide Sequence Alignment

Publications from Thesis and Abstract Presented at International Conferences

1. **Funwei RI**, Nderu D, N. Nguetse CN, Thomas BN, Catherine Falade C, Velavan TP and Ojurongbe O (2019) Molecular surveillance of pfhrp2 and pfhrp3 deletions in Plasmodium falciparum isolates and the implications for malaria rapid diagnostic tests in Nigeria. ***Acta Tropica*.https://doi.org/10.1016/j.actatropica.2019.05.016**

2. **Funwei RI,** Thomas BN, Falade CO, Ojurongbe O. (2018) Extensive diversity in the allelic frequency of Plasmodium falciparum merozoite surface proteins and glutamate-rich protein in rural and urban settings of southwestern Nigeria. Malar J. 2018 Jan 2; 17(1):1. doi: 10.1186/s12936-017-2149-5.

3. **7th MIM conference Dakar Senegal April 2018.**
   ***Paper presented: Comparative Evaluation of the Performance of Three Histidine Rich Protein- 2 (PfHRP-2) – Based Malaria Rapid Diagnostic Tests Against Microscopy in Ibadan, South West Nigeria for Guiding Malaria Treatment*. Roland Funwei (Presenter)**, Adebola Orimadegun, Obaro Michael, IkeOluwapo Ajayi, Ayodele Jegede, Olusola Ojurongbe, Olusegun Ademowo, James Ssekitooleko,Emanuel Baba E, & Catherine Falade.

4.Ninth EDCTP Forum Lisbon, Portugal 2018.

Paper presented: Detection of Plasmodium Falciparum Histidine rich Protein 2/3(Pfhrp-2/Pfhrp-3) Genes Deletion and Amino Acid Nucleotide Sequence Variability in Nigeria. **Roland Funwei(Presenter)**, David Nderu, Christian N. Nguetse, Bolaji N Thomas, Catherine Falade, Velavan TP and Olusola Ojurongbe: