IMMUNOLOGICAL STUDIES ON *Plasmodium falciparum* HISTIDINE RICH-PROTEIN2 (PfHRP2) GENE DELETION AMONGST SYMPTOMATIC MALARIA PARASITAEMIC PATIENTS IN KADUNA METROPOLIS

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BENIN CITY

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AND

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CERTIFICATION

This is to certify that this research work titled **"Immunological Studies on *Plasmodium Falciparum* Histidine Rich-Protein 2 Gene Deletion Amongst Symptomatic Malaria Parasitaemic Patients in Kaduna Metropolis"** was carried out by **Usman Itakure ABDULKADIR** with Matriculation Number **PG/BMS1613760** in the Department of Medical Laboratory Science, School of Basic Medical Sciences, College of Medical Sciences, University of Benin.

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DEDICATION

This research work is dedicated to the memory of my late mother Alhaja Halima Abdulkadir (1925-2023)

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ABBREVIATIONS

RDTs: Rapid Diagnostic Tests;

HRP2: Histidine Rich Protein2

CI: Confidence Interval

EG: Equatorial Guinea

CRP: C - reactive protein

IL-6: Interleukin 6

INF-γ: Interferon Gamma

HRP: Horse-radish Peroxidase

PCR: Polymerase Chain Reaction

M-PCR: Multiplex PCR

N- PCR: Nested PCR

NM- PCR: Nested Multiplex PCR

PfHRP2: Plasmodium falciparum Histidine-rich Protein 2

PfHRP3: Plasmodium falciparum Histidine-rich Protein 3

Pf- pLDH: Plasmodium falciparum Lactate Dehydrogenase

Pfdhfr: Plasmodium falciparum Dihydrofolate Reductase Gene

Pfdhps: Plasmodium falciparum Dihydropteroate Synthase Gene

Pfmdr1: Plasmodium falciparum Multidrug Resistant 1 Gene

Pfcrt: Plasmodium falciparum Chloroquine Resistance Transporter Gene.

ABSTRACT

Malaria is a major public health challenge worldwide with high morbidity and mortality. For prompt and accurate diagnosis, the World Health Organization (WHO) recommends rapid diagnostic test (RDT) as good alternative method for malaria diagnosis in sub-Sahara Africa prior to drug administration and treatment. Malaria RDTs are commercially available test kits and majority detects *Plasmodium falciparum* Histidine-rich protein-2 (PfHRP2 gene) as the target antigen. The WHO then recommends that PfHRP2 gene deletion must be monitored, especially in Africa. The aim of this study was to determine PfHRP2 gene deletion in blood samples of malaria parasitaemic patients in Kaduna metropolis. The Study was carried out using microscopy, PCR and RDT. All RDT-negative samples were further subjected to microscopy and molecular analysis (PCR) for malaria parasite speciation and PfHRP2 gene and deletion. Of the 1196 samples analysed, 694 were negative and 502 were positive by RDT. Of the RDT negatives, 83 samples were found to be microscopically positive, the 78 samples were for *Plasmodium falciparum* species and 5 non-*falciparum* species (2*P. Vivax*, 2*P. malariae*, 1*P. falciparum/Vivax*). These 83 samples were further subjected NM-PCR and were equally found positive for malaria, hence classified as RDT false–negatives. Statistical analysis for frequencies and confidence intervals (Cl) was used for prevalence estimates. Associations were assessed by chi square test. The level of significance was set at $P<0.05$. The significance package used was Minitab 22.1. From the 83 samples identified as RDT-false negative by PCR; 69 (5.8%) 95% Cl 4.6-7.0), had gene deletion both in PfHRP2 and PfHRP3, 4 samples (0.33%) 95% CI - 0.94-1.54) had deletion only in PfHRP2 but not in PfHRP3 and 5 samples (0.42%) 95% CI-0.84-1.64) had deletion in PfHRP3 but not in PfHRP2. With particular interest and considering PfHRP2 gene only within the total of 1196 samples 73(6.1%) 95% 4.76- 7.44) had evidence of deletion. This study provides evidence of PfHRP2 gene deletion in *Plasmodium falciparum* in Kaduna and PfHRP2 deletion was detected in the 73 samples analysed. This surveillance study also discovered that there is no significant difference ($p<0.001$) in Plasma level of CRP, IFN- and IL-6 of blood samples of participants with intact PfHRP2 gene and those with PfHRP2 gene deletion. It is, therefore, highly recommended that more surveillance studies across different geographical zone of the country be carried out to determine the full extent of PfHRP2 deletion frequencies. It also recommended that routine RDT kit contain additional PfHRP3 antigen, to improve the RDT efficacy and provide broad spectrum of diagnosis.

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CHAPTER ONE
INTRODUCTION

1.1 Background to Study

Malaria is a major public health challenge worldwide causing high annual morbidity and mortality particularly in malaria endemic areas of the world (Benito *et al*., 1994). The world health organization (WHO) recommends parasitological examination and confirmation for malaria parasite before drug administration and treatment. Malaria tests include microscopy, rapid diagnostic test (RDTs) and molecular analysis for the detection of malaria parasite in blood samples of parasitaemic patients (WHO 2021). The use of malaria RDTs is essential for prompt diagnosis of malaria that is used in a variety of contexts to overcome limitations of other diagnostic methods. The malaria RDTs use increases the availability and feasibility of accurate and prompt diagnosis (WHO 2021). The WHO also recommends rapid diagnostic tests as a good alternative method for malaria diagnosis especially in Sub-Sahara Africa where microscopic examination is not available (Boyle *et al.*, 2017). The malaria RDT is a commercially available test kits and majority detects P*lasmodium falciparum* histidine-rich protein 2 (*PfHRP2*) gene found in infected blood samples of malaria patients in several African countries (WHO, 2010). The main malaria control strategies include accurate and prompt diagnosis followed by effective treatment (Gerstl *et al*., 2010). In some research findings, reports indicate that there is *PfHRP2* gene deletion in blood samples of malaria parasitaemic patients collected in several African countries including Cameroon, Equatorial Guinea, Ghana, Kenya, Mali, Senegal, Rwanda in addition to India and South American countries. These gene deletions may cause false RDT-negative results (Amoah *et al*., 2016, Beshir *et al*., 2017, Bharti *et al*., 2016, Kozycki *et al*., 2017 and Parr *et al*., 2017). Based on this, the WHO

recommends that *PfHRP2* gene deletion must be properly monitored and surveyed, especially in Africa. The use of RDTs in the USA was approved in 2007 by the US department of Food and Drug Administration (FDA), but this approval was for use by hospitals and commercial laboratories, and not for use by clinicians, individuals or patients (CDC, 2020). It was also recommended that all rapid diagnostic tests are followed up with microscopy to confirm the species and quantify the proportion of the red blood cells that are infected (CDC, 2020). The use of RDTs may decrease the amount of time that is needed to determine whether a patient is infected with malaria hence the name 'rapid diagnostic test. The principle of Rapid diagnostic tests (RDTs) for malaria is based on principle of serological detection (an immunochromatographic test) of *Plasmodium falciparum* histidine-rich-protein2 found in the blood (erythrocyte) of patient infected with malaria parasites (WHO, 2016).

The recommended RDT of use for the most common and deadliest malaria parasite (*P. falciparum*) is the one that detects *Plasmodium falciparum* histidine-rich protein2 (FDA, 2007, WHO, 2016). This protein is the target antigen, and the principle of the test method is based on the immunochromatographic technique where PfHRP2 is the antigen present in the parasitaemic blood sample and RDT test strip which contains an immobilized corresponding antibody, this is an immunological test and therefore immunochemistry (WHO, 2016).

Immunological mediators such as C-reactive protein (CRP) and cytokine have been implicated in inflammatory processes and response in pathogenesis of malaria infection but their effect in Pfhrp2 gene deletion was not stated (Costa *et al*, 2020, Emmanuel *et al* 2019, and Sebina *et al* 2018). However, high levels of CRP and pro inflammatory

cytokines (IFN-γ and IL-6) have shown elevated plasma level in severe and uncomplicated malaria compared to the uninfected (Mbengu *et al*, 2016, Sebina *et al* 2017 and Emmanuel *et a*l, 2019)

1.2 Statement of Research Problem

Based on research findings, reports indicate that *PfHRP2* gene deletion was detected in several African countries including Cameroon, Equatorial Guinea, Ghana, Kenya, Mali, Senegal, Rwanda in addition to India and South America (Amoah *et al.,* 2016; Beshir *et al*., 2017; Bharti *et al.*, 2016; Kozycki *et al.* 2017; and Parr *et al.,*2017). This research is necessary to carryout surveillance studies to detect the presence of Pfrhp2 genes deletion in Kaduna metropolis, Nigeria and its implication on the use of RDT based test kits for laboratory diagnosis of malaria.

1.3 Justification of the Research Study

Since there is limited information on studies on *Plasmodium falciparum* histidine-rich protein 2 gene deletion in the area of study, Kaduna, to bridge this gap, it is therefore, necessary to carry out this survey in order to determine the presence of PfHRP2 gene deletion in the blood sample of RDT-negative malaria patients and the implication of the results obtained on RDT based tests (as recommended by WHO, 2010).

1.4 Aim of the Study

The aim of the study was to determine *Plasmodium falciparum* Histidine Ric0h-Protein2 (PfHRP2) gene deletion in blood samples of malaria parasitaemic patients in Kaduna metropolis.

1.5 Objectives of the Study

The specific objectives of this study are:

1. To determine why samples that were diagnosed as RDT-negative for malaria parasite was found to be positive by microscopy and PCR
2. To detect the presence of the target antigen, PFHRP2 genes in blood samples of the participants using molecular analysis.
3. To detect the presence and prevalence of PFHRP2 gene deletion in blood samples of participants that are RDT-negative (false-negative)
4. To determine whether PFHRP2 gene deletion is the cause of RDT false-negative results
5. To determine the serum levels of CRP, IFN-γ and IL-6 in malaria parasitaemic blood samples of participants with intact Pfhrp2 gene and those with gene deletion.
6. To highlight the significance of this surveillance to detect for PFHRP2 gene deletion responsible for RDT false-negative results and its implications on laboratory diagnosis of malaria.

1.6 Research Question

1. Is/are there sample(s) that are RDT-Negative but are diagnosed and confirmed to be positive by microscopy and PCR?
2. Is there any presence of PfHRP2 genes in the blood samples of the participants analyzed?
3. Is there any PfHRP2 gene deletion in the RDT-Negatives of blood samples participants analyzed?
4. Is PfHRP2 gene the cause of RDT false-negative results?
5. What is the prevalence of false RDT-negative results obtained from patients' blood samples due to PfHRP2 gene deletions?

6. Is there any difference between serum levels of CRP, IFN- γ and IL-6 in blood samples of participants with intact PfHRP2 gene and those with Pfhrp2 gene deletions?
7. Are there any significance of this survey and the implications of PfHRP2 gene deletions on the use of PfHRP2 RDT based test kit in the laboratory diagnosis of malaria?

1.7 Research Hypothesis

- **Null hypothesis:** There is no (absence) PfHRP2 gene deletion in the RDT-negative samples, therefore not responsible for false negative RDT results in the blood samples of the participants

CHAPTER TWO
LITERATURE REVIEW

2.1 Malaria

Malaria is the world most important parasitic disease of mankind with high morbidity and mortality. This result in over 241 million cases and 2 million deaths annually, the large majority of which is in sub-Saharan Africa (WHO, 2021). It is a major health problem that has been around for thousands of years and prevalent a11 over the world, but it is now confined to the tropical and subtropical areas of Asia, Africa, South and Central America. Although, nearly half of the worlds' population may be exposed to the risk of malaria (Cheesbrough, 2016). Malaria is a mosquito borne infectious disease that commonly affects humans and other animals. It is a life-threatening, preventable and curable disease, caused by the plasmodium species. Malaria parasite is transmitted to humans from the bites of infected female *Anopheles* mosquito, mostly in tropical countries. There five (5) parasites species and two of which are the deadliest; *Plasmodium falciparum* and *Plasmodium vivax* (Ochei and Kolhatkar, 2019)

2.2 History of Malaria

Malaria (or *ague*, as it was earlier called) has been known from ancient times as seasonal, intermittent fevers with chills and shivering, recorded in the religious and medical texts of ancient Indian, Chinese and Assyrian civilisations are believed to have been malaria. Charaka and Susruta have described the disease and ascribe its association with mosquitoes (Paniker, 2020). The great philosopher, Hippocrates in the ancient Greece in the 5th century BC gave an elaborate account of the clinical manifestation and observed the prevalence of the disease in certain areas and seasons. The association between the

disease and stagnant waters, swamps and marshy lands was recognised and measures taken to control the disease by effective drainage were practised in Rome and Greece by the 6th century AD. The name malaria (*mal*- bad, *aria*-air) was given in the 18th century Italians as it was believed to be caused by foul emanations from the marshy soil. Paludism, another name for malaria, also has a similar origin from *palus*, Latin for 'marsh'. The recent demonstration of a specific parasitic antigen in Egyptian mummies (preserved dead bodies) indicates that malaria was present thousands of years ago (Paniker, 2020)

In 1880, the specific causative agent of malaria was discovered in the red blood cells of a patient by Alphonse Laveran, a French army surgeon in Algeria. By 1886, Golgi in Italy described the asexual development of the parasite in red blood cells, erythrocytic schizogony, which therefore came to be called the Golgi cycle (Paniker, 2020). In 1891, Romanowsky in Russia developed a method of staining malaria parasites in blood films. Three different species of malaria parasites infecting man, *Plasmodium vivax, P. malariae* and *P.falciparum* were described in Italy between 1886 and 1890. The fourth species, *P.ovale* was discovered and identified only in 1922. The mode of transmission of the disease was established in 1897, when Ronald Ross in Secunderabad, India identified the developmental stages of malaria parasites in mosquitoes (Paniker, 2020).

These findings led to various measures for the control and possible eradication of malaria by mosquito control and both Ross (1902) and Laveran (1907) won the Nobel Prize for their discoveries in malaria (Paniker, 2020)

2.3 Malaria Parasite

Malaria parasites are protozoan parasite belonging to the subclass Coccidia, and genus Plasmodium. This genus consists of many species which causes malaria in mammals and birds (WHO, 2021). This parasite exhibits two cycles; the asexual cycle called schizogony which takes place in the erythrocytes (rbc) of vertebrates (intermediate host), and the sexual cycle called sporogony which takes place inside the mosquitoes (definitive host) (Ochei and Kolhatkar, 2019) There are four species of Plasmodium which can cause malaria disease in man. The species include Plasmodium *vivax, Plasmodium falciparum, Plasmodium malaria and Plasmodium ovale*. P. *falciparum*, and P. *vivax* are the widest spread, found mainly in the hotter and more humid regions of the world. It is the main species found in tropical (including Nigeria) and subtropical Africa (Paniker, 2020). The species *Plasmodium falciparum* contains several varieties of subtypes which show differences in geographical distribution, vector susceptibility, human infection pattern, drug susceptibility, morphology and antigenic composition (Cheesbrough, 2016).

Malaria is present in over 100 countries of the world, particularly in tropical and subtropical region. The highest burden is found in Africa followed by south Asia and Southeast Asia (WHO, 2021). Malaria is preventable and curable, but in the year 2022, there was an estimated 249 million cases and 608,000 malaria deaths in 85 countries, with the WHO African Region carrying a disproportionately high 94% of malaria cases and 95% of malaria deaths (WHO, 2022). Children under the age of five account for about 80% of all malaria deaths in the Region. Symptoms can be mild or life-threatening, with mild symptoms including fever, chills and headache, and severe symptoms including fatigue, confusion, seizures, and difficulty breathing. Infants, children under 5

years, pregnant women, travellers and people with HIV or AIDS are at a higher risk of severe infection (WHO, 2021). Malaria can be prevented by avoiding mosquito bites and with medicines, and treatments can stop mild cases from getting worse. There are five *Plasmodium* parasite species that cause malaria in humans, and two of these species *P. falciparum* and *P. vivax* pose the greatest threat, with *P. falciparum* being the deadliest malaria parasite and the most prevalent on the African continent. Four African countries account for over half of all cases of malaria deaths worldwide. Nigeria (26.8%, the Democratic Republic of Congo (12.3%), Uganda (5.1%) and Mozambique (5.1%), (WHO, 2021) Nigeria has the highest burden of malaria globally, accounting for nearly 27% of the global malaria burden (WHO, 2021). Also in 2021, the WHO reported 68 million cases and 194,000 deaths due to the disease (WHO, 2021). The risk of transmitting malaria exists throughout the country, all year round. However, the incidence of malaria is highest in the northern-western and north-eastern parts of the country. Malaria Consortium Nigeria has been working to improve malaria control, particularly in planning (WHO 2018). In 2020, it was estimated that 241 million cases of malaria occurred worldwide and about 627,000 deaths were recorded mostly in Africa (W.H.O). The findings also revealed that children under 5years, pregnant women, travelling to endemic areas, immigrants from endemic area and people with weakened immune system are the most affected and vulnerable. It was further reported that Nigeria constitutes 25% of total malaria deaths in Africa (CDC, 2016). The total funding for malaria control and elimination reached an estimated US S 2.7 billion in 2018 with contribution from government of endemic countries amounting to US S 900 million or representing 30% of total funds (WHO, 2021).

2.3.1 Malaria Parasite Species

There are five species of plasmodia cause malaria in man, *P. falciparum, P. malariae* P. *ovale P. vivax* and *P. knowlesi*. Two species, *P.vivax* and *P.falciparum* account for about 95 per cent of all malaria worldwide, the other two being of relatively minor importance. While these four species do not ordinarily infect animals, *P. knowlesi* was found in macaques (Collin, 2012).

Malaria parasites belong to phylum- Apicomplexa, class-Sporozoea, Order-Eucoccidea and Suborder-Haemosporina. The *P. vivax, P. malariae* and *P.ovale* are closely related to other primate malaria parasites while *P. falciparum* is more related to bird malaria parasites, and appears to be a recent parasite of humans, in evolutionary terms. This is the most likely reason; falciparum infection causes the severest form of malaria and is responsible for nearly all fatal cases (Amoah *et al*, 2016).

There are five species of Plasmodium parasites that can infect humans and cause malaria, this includes:

i. *Plasmodium falciparum*: This causes the most severe form of malaria, responsible for most malaria-related deaths. It is widely distributed in the tropics (Amoah *et al.,* 2016).

ii. *Plasmodium vivax*: This is the most widely distributed species, extending through the tropics, subtropics and temperate zones. It is characterized by causing recurrent malaria. (Kim *et al*., 2008 and Akiyama **et al**, 2018)

iii. *Plasmodium ovale*: This is a relatively rare species, found primarily in Central West Africa and some South Pacific Island (Mendes *et al*., 2011).

iv. *Plasmodium malariae*: Occurs sporadically in the different part of the world, it is the least severe form, causing a mild and chronic infection (Koita *et al*., 2012).

v. *Plasmodium knowlesi*: It is a zoonotic species, primarily infecting macaques in Southeast Asia, but since it can also infect humans, it is also disease a disease of medical importance (Collin, 2012).

2.4 Vectors

The human malaria is transmitted by the female Anopheles mosquito after taking a blood meal. The male mosquito feeds exclusively on fruit juices, but the female needs at least two blood meals before the first batch of eggs can be laid (Cheesbrough, 2016). Malaria parasites in other animals (apes, monkeys, rodents) are transmitted by *Anopheles*, but bird malaria parasites are carried by *Culex, Aedes* and other genera of mosquitoes (Cheesbrough, 2016).

2.5 Morphology and Life Cycle

The life cycle of malaria parasites comprises two stages: an asexual phase occurring in humans and the sexual phase occurring in the mosquito

2.5.1 Asexual Phase

In the asexual phase, the parasite multiplies by division or splitting, a process called schizogony (from schizo-to split and gone-generation). This asexual phase found in man it is also called the vertebrate, intrinsic or endogenous phase (Paniker, 2020). In humans, schizogony occurs in two locations: in the red blood cell called erythrocytic

schizogony and in the liver cells, exoerythrocytic schizogony or the tissue phase (Paniker, 2020). Since schizogony in the liver is an essential step before the parasites can invade erythrocytes, it is therefore called pre-erythrocytic schizogony. The products of schizogony, whether erythrocytic or exoerythrocytic, are called merozoites (meros-a part, *zoon*-animal) (Paniker, 2020).

The sexual phase takes place in the female Anopheles mosquito, even though the sexual forms of the parasite (gametocytes) originate in human red blood cells. Maturation and fertilisation take place in the mosquito, giving rise to a large number of sporozoites (from sporos-seed). Hence this phase of sexual multiplication is called sporogony. It is also called the invertebrate, extrinsic, or exogenous phase (Paniker, 2020)

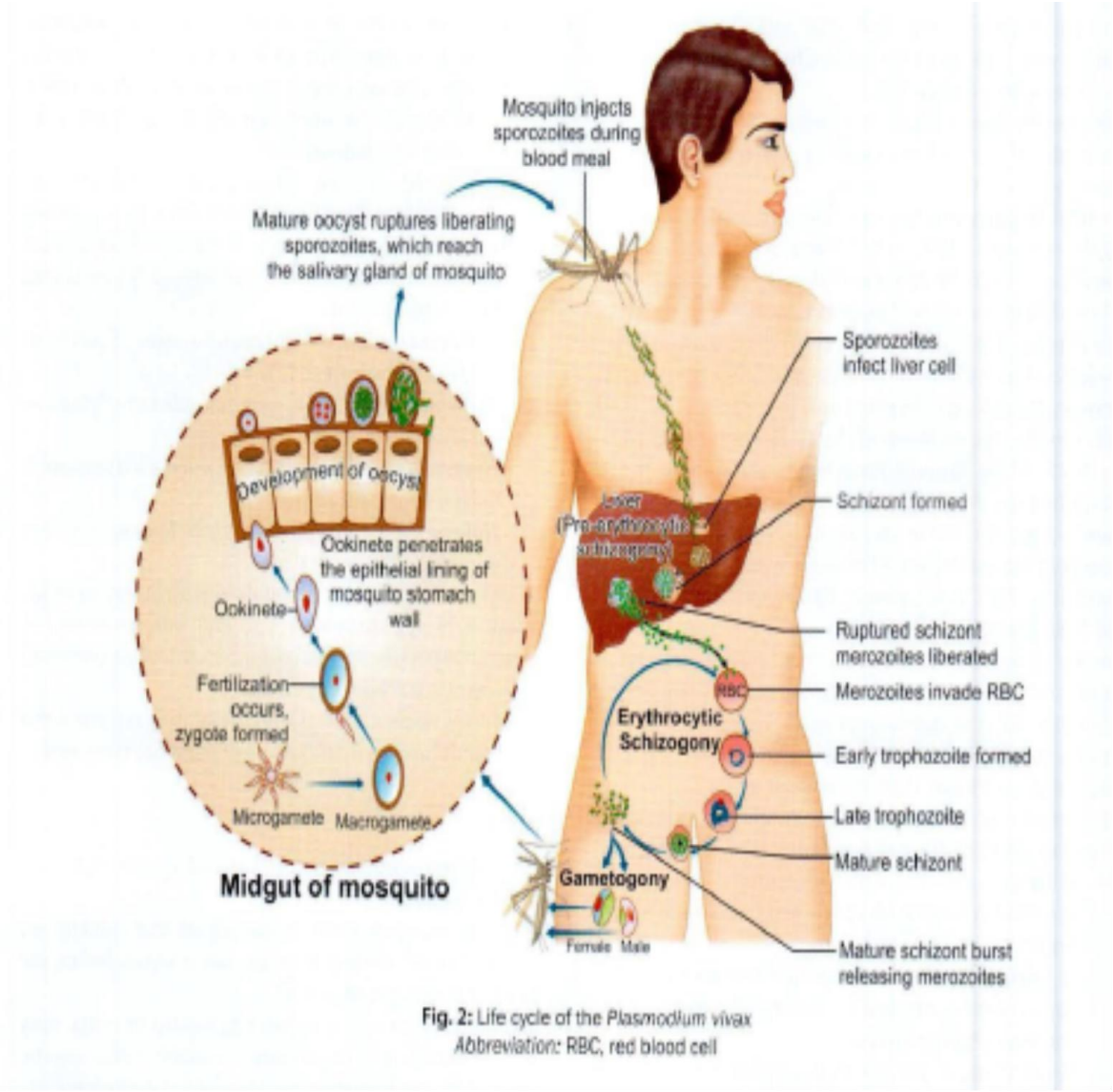

Fig. 2: Life cycle of the *Plasmodium vivax*
Abbreviation: RBC, red blood cell

Figure 2.1 Life Cycle of Malaria Parasite.

Source: Paniker (2020)

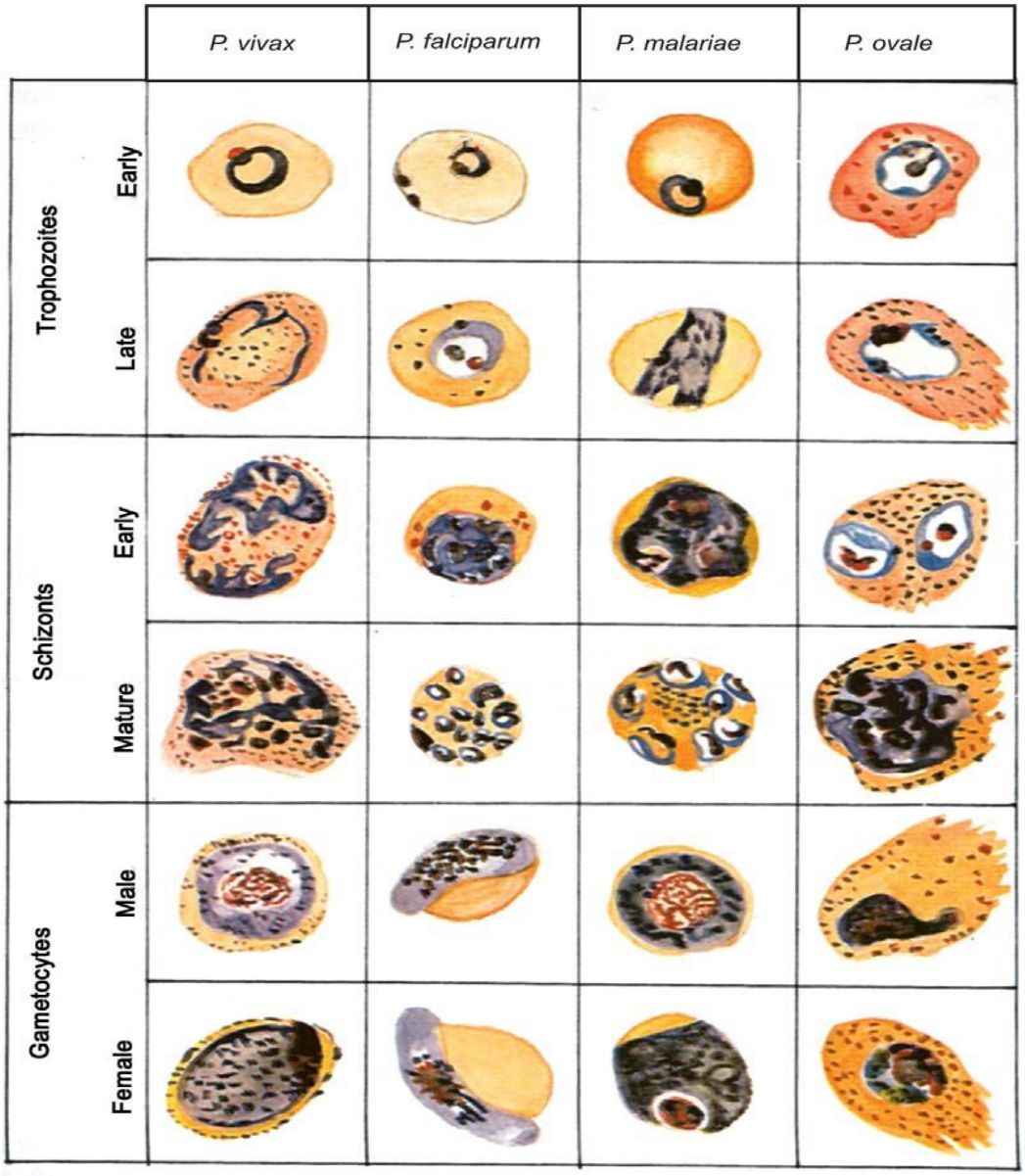

Figure 2.2: Malaria parasites—Erythrocytic stages of the four species (Giemsa stain. Magn × 2000

Source: Paniker (2020)

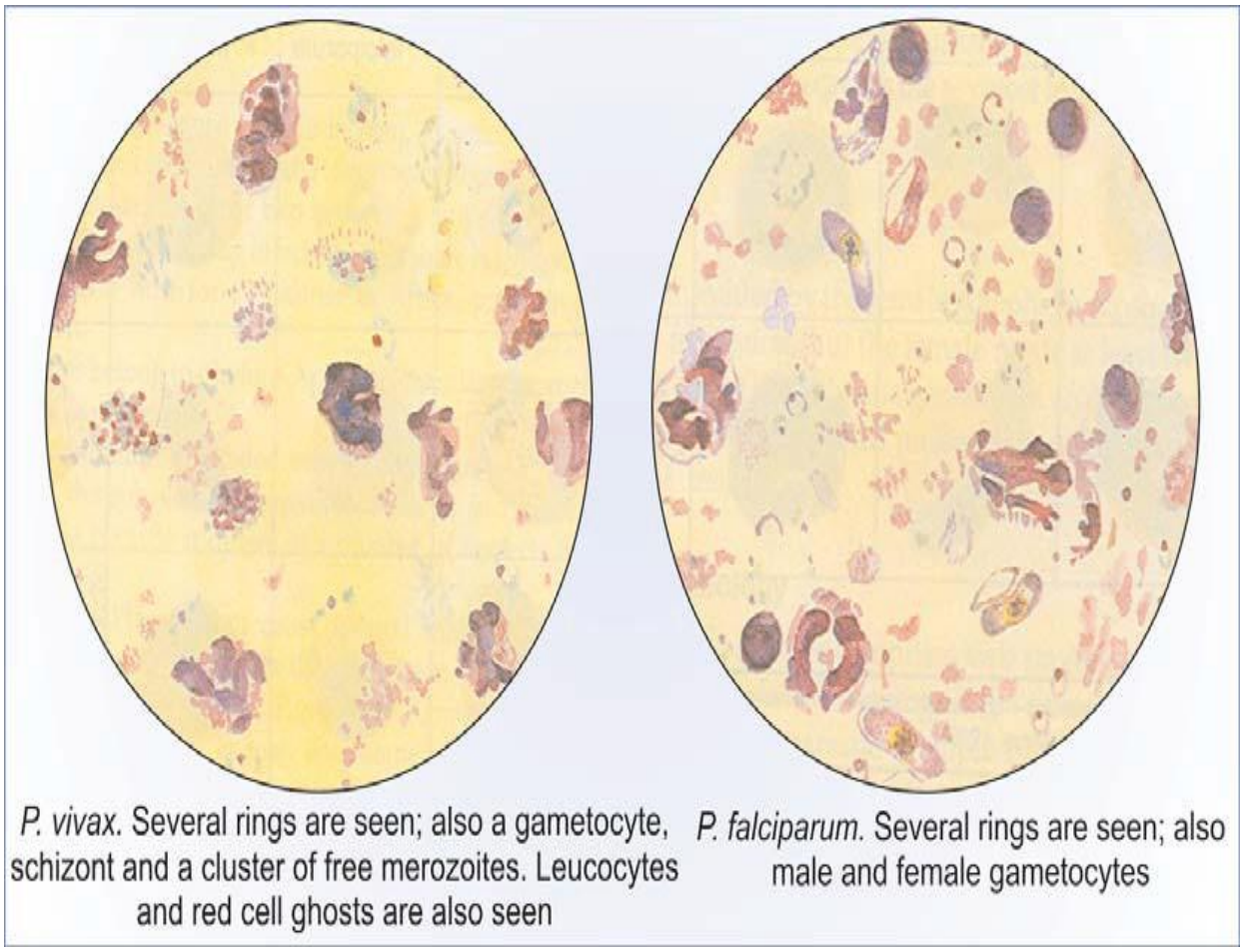

Figure: 2.3 Malaria parasites in thick blood smear (Giemsa stain, Magn. × 1000)

Source: Paniker (2020)

Thus, there is alternation of generations in the life cycle of malaria parasites asexual and sexual generations alternatively. Also, there in existence is alternation of hosts, as the asexual phase takes place in humans followed by the sexual phase in the mosquito. Hence, the complete life cycle of the malaria parasite comprises an alternation of generations with an alternation of hosts. As the sexual phase occurs in the mosquito, it is considered the definitive host of malaria parasites. The humans as the intermediate host since the human phase consists of asexual multiplication (Morley, 2012)

2.5.2 The Human Phase

The humans Infection is through the bite of the infective female Anopheles mosquito. The sporozoites which are infective forms of the parasite are present in the salivary gland of the mosquito. They are injected into blood capillaries when the mosquito (Rathnapala *et al.,* 2017)

2.5.3 The Mosquito Phase

When a female Anopheles mosquito obtains a blood meal and ingests parasitised erythrocytes, the asexual forms of malaria parasites are digested, but the gametocytes are set free and move to the stomach to undergo further development. Within 15 minutes of entry into the stomach of the mosquito, the male gametocyte divides into 8 nuclei, from each of which protrudes a long, actively motile whip-like filament. These flagella which are the male gametes (*microgametes*) lash about for a while and then break free. This process of formation of male gametes from the gametocyte is called *exflagellation*, (Paniker, 2020). This can take place outside the body of the mosquito also and can be observed under the microscope (WHO, 2023).

Exflagellation can be demonstrated under the microscope by making a thick film of

freshly drawn blood containing mature gametocytes on a microscopic glass slide and placing it in a warm moist chamber, such as a Petri dish containing filter paper soaked in warm water. It is then examined under the microscope after about 10 minutes, the male gametocyte can be observed and seen to shed its erythrocytic envelope and put forth up to eight slender active flagella containing nuclear material from the original nucleus. After detaching from the cell body, the flagella lash about vigorously in the plasma. At 25°C, the exflagellation is complete in 15 minutes for *P. vivax* and *P. ovale,* and 15 to 30 minutes for *P. falciparum* (Paniker, 2020). The female gametocyte does not divide but undergoes a process of maturation to become the female gamete or *macrogamete*. It is fertilised by one of the microgametes to produce the zygote. Fertilisation occurs in half to two hours after the blood meal (Baker, 2010).

The zygote, which is initially a motionless round body elongates and within 18 to 24 hours, becomes a vermicular motile form with an apical complex anteriorly. This is called the *ookinete* ('travelling vermicule'). It penetrates the epithelial lining of the mosquito stomach wall and comes to lie just beneath its basement membrane. It becomes rounded into a sphere with an elastic membrane. This stage is called the *oocyst* (Paniker, 2020). There may be up to several hundred pigmented oocysts in the stomach of a mosquito. The oocyst matures, increasing in size, with the nucleus undergoing multiple divisions. This *sporogony* leads to the development within the oocyst of about a thousand sporozoites, 10 to 15 μm in length, each with a central nucleus and an anterior apical complex. The mature oocyst which may be about 500 μm in size bulges into the body cavity of the mosquito, and when it ruptures the sporozoites enter the haemocoele. The sporozoites reach the salivary

glands situated in the thorax of the mosquito, penetrate the acinar cells and enter the salivary ducts. The mosquito is now infective and when it feeds on humans, the sporozoites are injected into the skin capillaries to initiate human infection (Fig. 5.4). The time taken for completion of sporogony in the mosquito is about 1 to 4 weeks; depending on the environmental temperature and the species (Trisnadi, 2020).

2.6 Mode of Transmission

The primary mode of transmission of malaria is through the bite of infected female Anopheles mosquitoes after a blood meal through the following root:

i. Vector transmission: Infected female Anopheles mosquitoes transmit malaria parasites (sporozoites) to humans through their saliva when they bite an individual to suck blood.

ii. Blood transfusion: In rare cases, malaria can be transmitted through blood transfusions from an infected blood donor.

iii. Organ transplantation: Malaria can be transmitted through organ transplantation from an infected organ donor.

iv. Mother-to-child transmission: Malaria can be transmitted from an infected mother to her child during pregnancy, childbirth, or breastfeeding (vertical transmission).

v. Contaminated needles: Sharing contaminated needles or syringes can transmit malaria.

vi. Laboratory exposure: Accidental needle sticks or cuts with contaminated instruments can transmit malaria in laboratory settings.

vii. Transplacental transmission: Malaria can be transmitted from an infected mother to her foetus during pregnancy.

viii. Blood-borne transmission: Malaria can be transmitted through blood-borne exposure, such as during surgical procedures or trauma (Ugah *et al*., 2017)

The immune system of certain individuals may be compromised due to immunosuppressive medication after organ transplant or HIV/AIDS treatment. Patients on immunosuppressive drugs face heightened vulnerability to malaria. Weakened immune system diminishes the body ability to resist malaria parasite, increasing the risk of severe and complicated malaria. Individuals who are exposed to malaria lack immunity to malaria and also travellers from non-endemic areas to endemic areas are at higher risk of malaria infection (Kotepui *et al.,* 2015).

2.7 Pathogenesis of Malaria

The incubation period normally varies from 8 to 40 days, being shortest in *P.falciparum* and longest in *P. malariae* infections. The average incubation periods varies from 8-11 days for falciparum, 10 to 12 days for vivax and ovale and 18 to 40 days for quartan malaria. However, very much longer incubation periods may proceed up to 9 months have been recorded with certain strains of *P. vivax* (*P. vivax hibernans*) (Paniker, 2020).

The incubation period can be distinguished from the prepatent period, which is the interval between the entry of the parasites into the host and the time when they first become detectable in blood. The minimum level of parasitaemia for their microscopic detection is called the microscopic threshold. This is about 50 to 100 parasites per microliter (uL) of blood (Minkah, 2018). Clinical disease develops only

later, when after several further cycles of multiplications, the level of parasitaemia rises high enough to cause fever, this is called fever threshold or pyrogenic density (Minkah *et al.,* 2018). The first clinical illness marking the end of the incubation period is called the primary attack. The typical manifestation of malaria consists of periodic bouts of fever with rigor, followed by anaemia and splenomegaly. True rigor is typically present in vivax malaria and is less common in falciparum infection. The febrile paroxysm comprises three successive stages. In the cold stage last for 15 to 60 minutes, here the patient experiences intense cold and uncontrollable shivering. This is followed by the hot (fever) stage, lasting for 2 to 6 hours, when the patient feels intensely hot. The temperature rises to 41°C or higher. Severe headache, nausea and vomiting are common. This is followed by the sweating stage, when the patient is drenched in profuse sweat. The temperature drops rapidly and the patient usually falls into deep sleep, to wake up refreshed. The paroxysm usually begins in the early afternoon and lasts for 8 to 12 hours. The periodicity of the attack varies with the species of the infecting parasite (Minkah *et al.,* 2018).

The periodicity is approximately 48 hours in tertian and 72 hours in quartan malaria. In quotidian periodicity, with the fever occur at 24-hour intervals and may be due to two broods of tertian parasites maturing on successive days, or due to mixed infections. Regular periodicity is seldom seen in the primary attack but is established usually only after a few days of continuous, remittent or intermittent fever (Kim *et al.,* 2020).

All clinical manifestations in malaria are due to the products of erythrocytic schizogony and the host's reactions to them. The exoerythrocytic liver cycle and

gametogony do not appear to contribute to clinical illness. The febrile paroxysms follow the completion of erythrocytic schizogony, when the mature schizont ruptures, releasing red cell fragments, merozoites, malarial pigment and other parasitic debris (Paniker, 2020). Macrophages and polymorphs phagocytose these and release large quantities of endogenous pyrogens, leading to elevation of temperature. Cytokines such as tumour necrosis factor (TNF) and interleukin-l may play a pivotal role in the pathogenesis of malarial fever (Siregar *et al.,* 2022).

Malaria is a common tropical and sub-tropical disease that is prevalent in Africa, where it is a major public health concern. The disease is transmitted through the bite of infected *Anopheles* mosquito during a blood meal. It is also transmitted through blood transfusion, placenta, and use of infected needle. The epidemiology of malaria is very complex and is influenced by factors such as environment, the parasite, the vector and human related factor.

2.8 Geographical Distribution of Malaria

Malaria is the most common and prevalent disease in the tropics and sub-tropical regions of the world. The Sub-Saharan Africa carries the highest burden of malaria and Nigeria accounting for over 25% of malaria cases (WHO, 2021).

The risk of malaria varies from one geographical region to another, with some areas experiencing all year-round transmission while others are seasonal outbreaks. Malaria Is also found in America, Asia, South-East Asia and Oceania (WHO, 2020).

The disease was once widespread but has been eliminated from many developed regions (especially developed countries) during the mid-20th Century (WHO, 2020).

The World Health Organization estimates that nearly 500 million malaria tests are performed annually and it recommended the use of parasite base diagnostic testing i.e. either microscopy or rapid diagnostic test before the administration of treatment. Result from parasitological confirmation can be available in 30 minute (WHO, 2021). Rapid and accurate diagnosis is integral to the appropriate treatment of affected individual and preventing further spread of infection in the community. Reference microscopic diagnosis and other specialized tests such as serology, PCR and drug resistance testing are also available (WHO, 2021). For a definitive diagnosis to be made, laboratory test must demonstrate the malaria parasite or their component (e.g. protein as antigen) as qualitatively detected by rapid diagnostic test kits (WHO, 2016).

2.9 Epidemiology Concepts of Malaria

- Endemicity: This refers to the presence of a disease in a population in a particular geographic region. Malaria can be endemic in areas where the disease is constantly present.
- Infection and Disease: When an individual is infected with malaria parasite (malaria parasite is present in blood) but not necessary showing signs and symptoms of malaria.
- Cases and Incidence: This is the number of new cases of malaria in a population in a specific period
- Case Prevalence: This is the total number of cases of a specific disease present in a population at a given time. This is the measure of disease burden in a community

- Case Fertility Ratio: This is the proportion of malaria cases that result in death in a given population.
- Surveillance: monitoring cases and transmission pattern is essential for identifying areas at high risk and tailoring preventive and control effort (WHO,2022).

2.10. Epidemiology of Malaria Disease in Africa

The estimated number of deaths stood at 59,700 in 2023 compared to 60000 in 2020 (WH0). Globally in 2023, there were an estimated 263 million malaria cases and 597,000 malaria deaths in 83 countries (WHO). This number was higher than 253 million cases reported in the previous year (2022).

The WHO African regional office reports that Africa carries disproportionate higher share of global malaria burden and is home to 94% of malaria case (246 million) and 95% (569 000) of malaria deaths. Children within age 5 accounted for about 76% of malaria deaths in the region (WHO,2022)

Overall, Nigeria accounts for 30.9%, Democratic Republic of Congo, 11.3%, Niger 5.9% and Tanzania 4.3%. These four countries account for over half of total malaria death ((WHO, 2023))

Nigeria carries the highest global burden of malaria, counting for about 68 million (27%) of global malaria cases and 198 000 (31%) of global malaria deaths (WHO, 2021). The disease is endemic throughout the country, with transmission varying seasonally in the

north and more consistently in the south. Children under five and pregnant women are particularly vulnerable (WHO,2021).

- **High Burden:**

  Nigeria bears a significant portion of the global malaria burden, both in terms of number of cases and deaths. There is high morbidity and mortality (Malaria Consortium, 2022)

Endemicity:

Malaria is present throughout the country, meaning it is consistently transmitted in all areas (Malaria Consortium, 2022).

- **Seasonal Variation:**

  Transmission rates are higher during the rainy season, particularly in the northern and north-eastern parts of the country (Malaria Consortium, 2022)

- **Vulnerable Groups:**

  Children under five and pregnant women are at higher risk of severe malaria complications (Malaria Consortium, 2022).

- **Transmission:**

  Malaria is primarily transmitted through the bites of infected female Anopheles mosquitoes (Malaria Consortium, 2022).

- **Vector Species:**

  The primary malaria vectors in Nigeria are Anopheles *coluzzii* and Anopheles (An.) gambiae s.s (Malaria Consortium, 2022)

- **Parasite Species:**

  Plasmodium falciparum is the predominant species causing malaria in Nigeria (WHO,2021).

- **Socio-economic Factors:**

  Poverty and lack of access to preventative measures like insecticide-treated nets (ITNs) can increase the risk of malaria (Malaria Consortium, 2022).

- **Spatial Variation:**

  Malaria incidence can vary across regions, with higher rates observed in rural areas and certain geographic zones like the Northwest and Southeast (WHO, 2022).

2.11 Malaria in Kaduna

Malaria prevalence in Kaduna Metropolis, Nigeria, is significant, particularly among children under five. Studies have shown a prevalence rate of 46.5% among febrile patients in Kaduna Metropolis. The rainy season, typically from April to October, is associated with higher malaria transmission rates, with peak transmission between June and October. Rural areas and specific LGAs like Kaduna-South have higher malaria incidence (Malaria Consortium, 2022).

High Prevalence: Malaria remains a major public health issue in Kaduna State, with studies reporting high prevalence rates, particularly in children under 5 years old (Malaria Consortium, 2022).

Seasonal Variation: Malaria transmission is strongly linked to the rainy season. The wet months, particularly August, see higher prevalence (Malaria Consortium, 2022).

Age-Related Incidence: Children under 5 are most affected, with the 12-59 month age group being particularly vulnerable (Malaria Consortium, 2022).

Geographic Variation: Specific LGAs within Kaduna State, like Kaduna-South, have higher malaria incidence (Malaria Consortium, 2022).

Factors Associated with Malaria: Rural areas, compound houses, and lower socio-economic status have been linked to higher malaria prevalence (Malaria Consortium, 2022).

Surveillance Data: Routine surveillance data in Kaduna State shows a downward trend in malaria cases, but the problem persists (Malaria Consortium, 2022).

2.11.1 Factors Contributing to Malaria Prevalence

Environmental Factors: Rainfall, temperature, and humidity create conditions favorable for mosquito breeding and transmission (Malaria Consortium, 2022).

Social and Economic Factors: Poverty, poor sanitation, and lack of access to clean water and housing can increase malaria risk (Malaria Consortium, 2022).

Behavioral Factors: Lack of knowledge about malaria prevention and treatment, delayed care-seeking, and reliance on unproven treatments can exacerbate the problem (Malaria Consortium, 2022).

Access to Healthcare: Limited access to effective diagnostic tools and treatments, particularly in rural areas, can hinder malaria control efforts (Malaria Consortium, 2022).

2.11.2. Malaria Control Strategies

Prevention: Interventions like insecticide-treated bed nets (ITNs), indoor residual spraying (IRS), and vector control measures are crucial for reducing malaria transmission (WHO, 2021)

Early Diagnosis and Treatment: Rapid Diagnostic Tests (RDTs) and timely access to effective antimalarial drugs are essential for managing malaria cases (Malaria Consortium, 2022).

Community Mobilization: Raising awareness about malaria prevention, treatment, and care-seeking behaviors is important for improving community health outcomes (Malaria Consortium, 2022).

2.11.3 Addressing Malaria in Kaduna

Targeted Interventions: Focusing on high-risk areas and populations, such as children under 5 and those in rural settings, is crucial for effective malaria control (Malaria Consortium, 2022).

Improved Access to Healthcare: Ensuring that all residents have access to quality healthcare services, including malaria diagnosis and treatment, is essential (Malaria Consortium, 2022).

Community-Based Health Workers: Empowering community health workers to deliver malaria prevention and treatment services can improve access and coverage (Malaria Consortium, 2022).

Data-Driven Decision-Making: Utilizing surveillance data to inform malaria control programs and tailor interventions to specific needs is important for achieving progress (Malaria Consortium, 2022).

2.12 Malaria Risk Factors

The understanding of factors that contribute to the susceptibility of malaria infection is crucial for the effective prevention and control strategies. The vulnerability to this infection depends on several risk factors, these include:

2.12.1 Immune Status

The immune system of certain individuals may be compromised due to immunosuppressive medication after organ transplant or HIV/AIDS treatment. Patients on immunosuppressive drugs face heightened vulnerability to malaria. Weakened immune system diminishes the body ability to resist malaria parasite, increasing the risk of severe and complicated malaria. Individuals who are exposed to malaria and also travellers from non-endemic areas to endemic areas are at higher risk of malaria infection (Kotepui *et al*, 2025

2.12.2 Geographical Factors

- **Warm and Humid Areas**: Living in warm and humid areas, especially malaria-endemic regions, increases the risk of malaria (WHO, 2021).
- **Proximity Water Bodies:** Areas with water bodies such as marshes, swamps, lakes, or rivers serve as breeding grounds for mosquitoes and increases susceptibility to malaria (WHO, 2021).
- **Dense vegetation:** Thick vegetation provides an ideal environment for mosquitoes to thrive, increasing the risk of malaria (WHO, 2021)
- **Rural and Urban Areas**: Areas with limited resources and poor sanitation especially in rural areas are more prone to malaria than well-developed urban areas. However, urban areas with high population density can also contribute to malaria transmission by providing ample hosts for mosquitoes (CDC, 2020). Poor housing and sanitation, such as inadequate drainage and sewage disposal, combined with a lack of insecticide-treated nets, exacerbate vulnerability (WHO, 2021).

- **Climate Change:** Alteration of weather patterns may impact mosquito populations and malaria transmission (WHO, 2021)
- **Environmental changes**: Changes such as deforestation or irrigation projects, alters the environment and create new breeding grounds for mosquitoes to thrive, while seasonal variations, particularly the rainy season, peak mosquito breeding in tropical regions (WHO, 2021).
- **Geographical Isolation**: Isolated or remote areas may encounter challenges in finding access to health care services and implementing control measures.
- **Local or International Travel:** Travel to malaria-endemic areas either local areas or international increase the risk of importing or spreading the risk of malaria (WHO, 2021).

2.12.3 Socioeconomic factors

Economic situation like poverty and limited access to healthcare facilities can worsen outcomes due to delayed diagnosis and treatment. The community that can afford hospital expenses, clean environment, mosquito control and other factors are more vulnerable to malaria. Socioeconomic implication of malaria burden is perpetuation of poverty causing significant economic loses, hampering productivity and impeding socio-economic development in affected communities (WHO, 2021).

2.12.4 Age Factor

Certain populations are more vulnerable to malaria:

- **Children under 5 years old:** They are more susceptible due to their underdeveloped immune systems.

- **Pregnant women:** Hormonal and immunological changes make them more prone to malaria compared to non-pregnant women.
- **Immunocompromised patients:** Those with conditions like HIV or organ transplants, or individuals on immunosuppressive drugs, are at heightened risk.
- **Immigrants and travellers:** Individuals traveling to malaria-endemic areas are at increased risk due to lack of immunity (WHO, 2010).

2.12.5 Genetics and Malaria Vulnerability

Genetic factors play important role in determining an individual susceptibility to malaria. The genetic trait affects the ability of malaria parasite to invade host blood and replicate within the red blood is cells. However, the genetic diversity within the malaria parasite population may influence the severity and course of the disease (Amambua-Ngwa, 2012).

2.12.6 Occupation and Activities

Health workers like doctors, nurses, agricultural and construction workers are at higher risk of malaria infection. These occupations often involve regular and significant exposure to mosquito bite in malaria endemic areas. Working during peak mosquito activity hours with inadequate protective measures and proximity to mosquito breeding sites contribute to heightened susceptibility. Outdoor activities such as camping or hiking in malaria endemic areas increase risk of malaria infection (D'Souza and D'Souza, 2012).

2.12.7 Prophylaxis and Therapy Failure

Malaria treatment failure may occur from both prophylaxis and treatment which is a serious issue in malaria management. When antimalarial drug fail to eradicate the

parasite or prevent recrudescence while prophylaxis failure occurs preventative medication fail to stop infection (Ashley *et al.,* 2014).

The factors contributing prophylaxis failure include:

- **Drug resistance:** which occur when malaria parasites develop resistance to antimalarial drug rendering prophylactic treatment ineffective.
- **Missed Doses:** Non- compliance with drug prescription by skipping doses of preventive drug; either daily or weekly regimens can lead to inadequate bioavailability in blood levels and failure.
- **Inadequate Dosing:** Taking under dose of a drug can result in prophylactic failure
- **Drug Interaction:** Some drugs can interfere with antimalarial drugs through drug interaction thereby reducing the effectiveness of the drug and potentially leading to prophylactic failure.
- **Duration of Prophylaxis:** Taking recommended duration of drug during travelling to malaria endemic reduce the risk of malaria (Malaria Consortium, 2022).

2.12.8 Therapy Failure.

- **Drug Resistance:** Drug resistance can render antimalarial drugs ineffective in eradicating the parasite.
- **Inadequate Dosing:** Taking the wrong dosage (under dose) will cause inadequate bioavailability of the drug in blood cause treatment failure

- **Poor Dosage Compliance:** Not taking the full course of antimalarial drugs for the prescription can lead to incomplete treatment and treatment failure.
- **Quality of Drugs:** Substandard or counterfeit antimalarial drugs can cause treatment failure
- **Timing of Treatment:** Taking treatment when malaria infection has progressed to a severe stage may increase the risk of treatment failure (Malaria Consortium, 2022).

2.12.9 Poor Health Facility

Living in areas with inadequate and poor healthcare infrastructural and manpower needs makes it difficult to receive prompt and effective treatment. The infrastructural needs include adequate clinic accommodation and diagnostic materials. Manpower includes all the healthcare provider to diagnose, manage and treat malaria (WHO, 2021)

2.12.10 Surveillance and Control

Malaria surveillance involves early detection, management, and treatment of cases to reduce morbidity and mortality. Effective vector control measures include proper drainage systems, the provision of insecticides or larvicides, and the widespread use of insecticide-treated nets. Elimination of mosquito breeding and transmission areas is a key component of malaria control strategies (CDC, 2020; WHO, 2021).

2.13 Immunity in Malaria

The malaria immunity is a complex process that involves both natural and acquired immunity. The resistance against malaria infection develops against *Plasmodium* parasite the causative agent of malaria. The immunity is overwhelmed and cannot

completely eliminate the parasite but reduces the severity of the illness and frequency of infection. With repeated exposure, it develops over time and influenced by age, genetics and co-infections (Riley and Stewart, 2013)

2.13.1 Natural Immunity

Natural (innate) immunity against malaria is seldom known, but few naturally occurring episodes illustrate its importance. Red cells invasion by merozoites is facilitated by the presence of specific glycoprotein receptors on the erythrocyte surface (O'Flaherty and Benson, 2020). It has been discovered that individuals who lack the Duffy blood group antigen (Fya Fyb) are resistant to infection by *P. vivax*. This Duffy antigen appears to be the receptor for the malarial parasite and this antigen is absent in the native population of West Africa (O'Flaherty and Benson, 2020). This might be the main reason why *P.vivax* malaria is not prevalent in sickle cell anaemia because *P. vivax* does not multiply properly in red cells containing the abnormal haemoglobin S. In Africa where *falciparum* infection is hyper endemic, sickle cell trait is very common. It has been hypothesized that the sickle cell trait, which is otherwise undesirable, tends to be conserved there because of the survival advantage it offers in falciparum malaria. Haemoglobin F is the foetal haemoglobin which is present in neonates provides them immunity against malaria (Luzzatto, 2012).

Glucose-6-phosphate dehydrogenase (G6PDH) deficiency found in the Mediterranean coast, Africa, the Middle East and India provide innate immunity to malaria. HLA-B53 is also associated with protection from malaria (Leslie *et al.,* 2020).

Findings have shown that severe malnutrition and iron deficiency may confer some protection against malaria (Luzzatto, 2012). In severe famine as was observed in North

Africa, malaria was rare, but on providing food and iron supplements, the patients began to manifest clinical malaria. In pregnancy, *P. Falciparum* malaria is more severe particularly in the first trimester, and may be enhanced by iron supplementation (Sow *et al.,* 2015)

The spleen appears to play a major role in immunity against malaria, hence an individual that have undergone splenectomy more susceptibility to malaria (Sow *et al*, 2019)

2.13.2 Acquired Immunity

When an individual is infected with malaria parasites, the infection induces specific (acquired) immunity which can bring about clinical cure, but not complete elimination of the parasites from the blood. This acquired immunity can also prevent superinfection but is not effective enough to prevent re-infection. This state of immunity in an infected host is associated with continued asymptomatic parasitic infection, this is called premunition. The host is resistant to fresh infection (superinfection) as long as the pre-existing infection continues even though in subclinical form. But once the infection is eradicated from the blood, the immunity does not persist for long and cannot prevent subsequent infection (re-infection) (O'Flaherty and Beeson, 2022).

Acquired immunity is evident in endemic areas where infants below the age of 3 months are conferred with immunity by passive maternal antibodies. Children are highly susceptible to malaria; however, as they grow up they acquire immunity through subclinical or clinical infections so that the incidence of malaria is low in older children and adults (O'Flaherty and Beeson, 2022).

The antigenic fractions of malaria parasites of the four species of human parasites have both common as well as species-specific antigens. In the domain of each species, the different stages in the life cycle have stage-specific antigens. Immunity appears to be strain-specific, and one infection may not confer immunity against infection by a different strain of the same species of the parasite (Apinjoh *et al.,* 2015). Nevertheless, in endemic areas, repeated infections by multiple strains may confer broad spectrum of immunity (Apinjoh *et al.,* 2015).

Experimental findings on immunization against malaria date back to early in the 20th century. This study shows that injection of erythrocytic parasites with Freund's complete adjuvant was shown to elicit immunity in monkey malaria, but this was not practically applicable in humans. Although, only recently, after persistent and successful culture of malaria parasites, the availability of monoclonal antibodies and the development of cloning techniques that significant progress was achieved in this field. Several possibilities are being tested for the immunoprophylaxis of malaria (Apinjoh *et al.,* 2015).

Immunization against the sporozoites antigens could inhibit the first step in human infection thereby blocking the invasion of liver cells. Also antigenic surface component has been identified on the sporozoites and this sporozoites antigen ('circumsporozoite protein') has been cloned and its immunodominant epitope identified (Aboul-Ella *et al.,* 2024). The epitope sequence which consists of a small number of amino acids has been chemically synthesized and the gene has been introduced into the vaccinia virus and the recombinant virus has been shown to produce the sporozoite antigen (Aboul-Ella *et al.,* 2024).

Some other antigens have been considered as potential vaccines, including the merozoite surface protein-l (msp1), apical membrane antigen, erythrocyte binding antigen, a soluble antigen released during rupture of parasitised erythrocytes and a zygote antigen Pfs 25.SPf66 is a synthetic malaria vaccine that has undergone several field trials is developed by Manuel Patarroyo in Columbia. This is a synthetic peptide containing the amino acid sequences of three *P. falciparum* merozoite proteins linked together by a tetrapeptide from the circumsporozoite protein. The field trials in South America and Tanzania showed moderate protection, but much less effective in Gambia and Thailand (Wu *et al.,* 2022).

A method of inhibiting mosquito transmission has been proposed, by immunizing malaria patients or carriers with vaccine containing gamete or zygote antigens. When mosquitoes feed on them, the antibodies sucked in along with gametocytes prevent sporogony taking place; this method has been termed transmission blocking immunity. An ideal malaria vaccine should be one inducing multistage, multivalent, multi- immune responses. However, no appreciable scientific breakthrough is available at present. Much work is being done in developing DNA vaccines to meet a11 these requirements (Roeffen *et al.,* 2015).

Cell-mediated immunity is applicable in malaria, but little is known about its scope and importance and malaria does not appear to be aggravated by AIDS (Roeffen *et al*, 2015)

2.13.3 Immunopathology

Malaria parasite infection is known to cause some depression on the immune system. It has been suggested that immune depression caused by endemic malaria is responsible

for the Burkitt's lymphoma seen in many African children. While the Epstein-Barr virus causes asymptomatic infection or infectious mononucleosis in immunocompetent persons, and in African children whose immune system is severely compromised by recurrent malaria infection, the virus leads to lymphoma (Stone *et al.,* 2018).

Parasitised erythrocytes may undergo antigenic changes, which may lead to autoimmune phenomena. Immune complexes occur in malaria which may lead to nephropathies (Stone *et al*, 2018).

2.13.4 Immunopathogenesis of Malaria

During the blood infection stage, in response to the presence of the parasite, the host's immune system releases several proinflammatory molecules including IL-1β, IL-6, IL-8, IL-12 , IFN-γ, and TNF, all cytokines which play a defining role in controlling the parasite's growth and elimination. Regulatory cytokines such as transforming growth factor- (TGF-) β and IL-10 maintain the balance between the proinflammatory and anti-inflammatory responses. When this balance is disrupted, the exaggerated proinflammatory response leads to significant adverse effects associated with severe forms of malaria and a high mortality rate (Dobbs *et al.,* 2020*).*

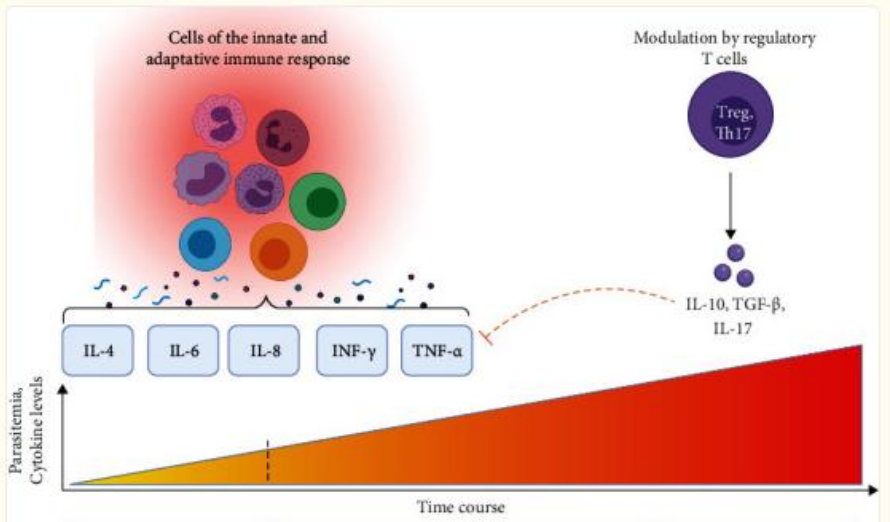

Figure 2.4: The role of different effectors cells and cytokine mediators involved in the pathogenesis of malaria

Cytokine production can be influenced by several factors. A recent study has shown that the polymorphism of TLRs affects the synthesis of cytokines in *Plasmodium vivax* malaria. For instance, subjects with the minor alleles of TLR4 (A299G), TLR6 (S249P), and TLR9 (-1486C/T) were detected to have lower amounts of IL-6 and IFN-γ. Although these cytokines play a defining role in eliminating the parasite, elevated levels are correlated with disease complications. In addition, the study showed that polymorphisms in the TLR4 (A299G), TLR6 (S249P), and TLR9 (-1486C/T) genes are associated with a lower synthesis of IL-10, an important modulator of the immune response. T/T genotype of the TLR9 polymorphism (-1486C/T) is correlated with elevated levels of IL-2 (Costa *et al.,* 2018).

Hemmer *et al* (2006) have shown a more potent host response per parasitized erythrocyte in infections caused by *Plasmodium vivax* or *ovale* than in those attributable by *Plasmodium falciparum*. However, a recent study on vivax malaria has revealed higher IL-10/TNF-α, IL-10/IFN- γ, and IL-10/IL-6 ratios, and similar inflammatory cytokine responses per parasitized erythrocyte, when compared to falciparum malaria. Additionally, carriers of very low *P. vivax* parasitaemia had considerably reduced levels of proinflammatory and regulatory cytokines when compared to patients with clinical manifestations of *P. vivax* malaria (Gonçalves *et al.,* 2012). These data draw attention to the fact that reaching the parasite density threshold is probably necessary to activate a host immune response (Gonçalves *et al.,* 2012).

Scherer *et al* (2016) suggest that blood viscosity is higher in patients with malaria and ultimately influences cytokine levels. In the serum of patients with *P. vivax* infection,

higher levels of IFN-γ and IL-17 and lower levels of TGF-β were determined. In addition, the study showed that incubation of blood collected from infected patients in the presence of IL-17 or IL-17 and IFN-γ led to the normalization of blood viscosity, being similar to that of uninfected individuals. The authors suggest that increasing serum IL-17 levels in malaria patients could be considered a host adaptation mechanism to control changes in blood viscosity, and IL-17 could thus be used as an immunomodulatory agent. IL-17 appears to act on erythrocytes by remodelling their cell membrane; it is well-known that erythrocytes in malaria are very sensitive to osmotic shock (Scherer *et al.,* 2016).

Role of TNFα- in Malaria Pathogenesis

TNF-α plays a pivotal role in increasing phagocytic uptake of parasites, being involved in parasite control (Gbedande *et al.,* 2020). In endemic areas, *P. falciparum*-specific CD4+ T cells coproducing IFN-γ and TNF-α were detected in patients who underwent *P. falciparum* infection (Boyle *et al.,* 2015). Consecutively, elevated serum TNF-α levels have been reported to directly correlate with the severity of *P. falciparum* malaria. Moreover, recent research states the role of TNF in lethal malaria forms (Boyle *et al.,* 2015). A systematic review that included 34 studies showed that elevated levels of TNF-α could be associated with cerebral malaria caused by *P. falciparum*, but the results are inconsistent. Most studies included a relatively small number of patients; further research is required (Leão *et al.,* 2020). In addition, there is a link between polymorphism within the TNF promoter region, parasitaemia, and malaria severity. Nguyen *et al* (2017). have shown that there is a correlation between symptomatic maximum parasitaemia and TNF-308, TNF-238, and TNF-244. Furthermore, there is an association between the number of

mild malaria episodes and TNF-244. A recent study presents an interesting finding. The study shows that TNF leads to increased intracellular calcium levels and decreases the count of intracellular parasites, using calcium as the second messenger of the pathway. The same research shows decreased expression of PfPCNA1, which encodes the *Plasmodium falciparum* proliferating-cell nuclear antigen 1, following treatment with TNF (Cruz *et al.,* 2016).

Role of IFN-*γ in* Malaria Pathogenesis

IFN-*γ* plays a dual role in the pathogenesis of malaria. A recent review of the role of IFN-*γ* in malaria suggests that type I IFN signalling limits CD4+ T helper cell activity during the blood infection stage. On the contrary, it is known that type I IFNs stimulate the release of proinflammatory cytokines, participating in the control of infection. The effect of type I IFNs depends on parasite factors as well as host factors (Sebina and Haque, 2018). The protective effect occurs especially when the level increases early in the evolution of the infection. However, chronic high levels will often lead to immune suppression (He *et al.,* 2020). Levels of type I IFNs are influenced by processes such as ubiquitination, phosphorylation, and ADP-ribosylation of the molecules involved in type I IFN pathways. IL-6 has a stimulating effect by increasing STAT1 expression, but there are also several other compounds with immunosuppressive effects, including suppressor of cytokine signaling 1 and 3 and ubiquitin carboxy-terminal hydrolase 18. In addition, molecules in the parasite structure may exert a regulatory effect on host type I IFN response (He *et al.,* 2020). Lourembam et al. have analyzed 58 patients infected with *P. falciparum* who had been divided according to WHO criteria into two groups with complicated and uncomplicated malaria. In the complicated malaria lot, higher levels of

IFN-γ and TGF-β and lower levels of IL-2 and IL-12a were reported when compared to the uncomplicated malaria cases. Moreover, the authors suggest that T cells are not a source for elevated levels of IFN-γ and draw attention to the need for further investigation of cells responsible for the exaggerated production of IFN-γ in cerebral malaria (Lourembam *et al.,* 2013).

Role of IL-6 and IL-8 in Malaria Pathogenesis

IL-6 and IL-8 are proinflammatory cytokines that may be involved in the pathogenesis of malaria. Sebina *et al* (2017). have shown that IL-6 has several roles in malaria; IL-6 participates in immunoglobulin synthesis, promotes the expression of inducible T cell costimulator (ICOS) in the Tfh cells, and activates the differentiation of B cells. Although IL-6 is an important factor of humoral immunity, the authors suggest that IL-6 appears to be nonessential in the control of *Plasmodium* infection and is only involved in the early stages of infection. Mbengue et al (2016) considered that elevated levels of TNF-α and IL-6 could be regarded as markers for severe malaria. Moreover, elevated levels of IL-8 have been identified in malaria patients, and a correlation between IL-8 and disease severity has also been noted. *P. falciparum* has the ability to produce a functional histamine-releasing homologous factor that promotes IL-8 release from neutrophils (Mbengue *et al.,* 2016). It has been reported that IL-8-251T/A promoter polymorphism is correlated with an increased risk of developing complicated malaria (Mahanta *et al.,* 2014). Rodrigues-da-Silva *et al* (2014). have revealed elevated levels of TNF-α, IFN-γ, IL-6, IL-8, IL-10, and IL-17 during the acute phase of malaria, and the high levels persisted through convalescence with the exception of IL-10 . According to a study performed by Otterdal *et al.* (2020), IL-27 levels are higher in patients with malaria than

in healthy subjects. A positive correlation was also observed between IL-27, *P. falciparum* parasitemia, and von Willebrand factor, but no impact on disease outcome was observed. IL-27 could be seen as an immunoregulatory cytokine in malaria, having a proinflammatory (induces IL-6 release) as well as an anti-inflammatory (inhibits IL-8 release) role.

Role IL-10 in Malaria Pathogenesis

IL-10 can be both a friend and an enemy, depending on the immune response and the type of parasite (Nakamae *et al.,* 2019). Nakamae *et al*, (2020) also have revealed that IL-10 inhibits protective immune responses against secondary infection with heterologous *Plasmodium* parasites. They have used infection models of *P. chabaudi chabaudi* (Pcc) and *P. berghei* ANKA (PbA) to observe the inhibition of IL-10 in association with increased CD4+ T cell activity, the release of IFN-γ, and decreased parasitaemia. A recent study found a correlation between *P. falciparum*-specific IL-10-positive T cells (IFN-γ- TNF-) and the risk of clinical malaria once infected (Boyle *et al.,* 2017). Requena *et al*, (2015) have compared the frequencies of T cell subsets among pregnant and nonpregnant women in a malaria-endemic area versus in a malaria-free zone. They have demonstrated that nonspecific proinflammatory responses at the first antenatal visit were associated with protection against *P. falciparum* malaria at delivery. Elevated intracellular levels of IL-10 in CD4+ T cells had a protective effect against *P. falciparum* infection and on haemoglobin levels at delivery. The plasma levels of IL-10/TNF-α and IL-10/IFN-γ ratios correlate with *P. vivax* concentrations in the blood (Gonçalves *et al.,* 2014). Sukhbaatar *et al*, (2020) have revealed that IL-10 production in CD4+ T cells is modulated by IL-27 in chronic *Plasmodium chabaudi* infection and have

focused on the pivotal role of the two cytokines in modulating pro- and anti-inflammatory responses in *Plasmodium* infections. *Plasmodium* may promote decreased IL-10 expression by downregulating GATA3 expression, resulting in poor control of the inflammatory process (Mahanta *et al.,* 2018).

Role of IL-4 in Pathogenesis of Malaria

The role of IL-4, an important regulator of Th2 responses, is incompletely elucidated in malaria. IL-4 induces Th2 responses and limits both the inflammatory process and Th1 responses, being associated with a protective role in severe forms of malaria (Wu *et al.,* 2022). Wu *et al,* (2022) also evaluated the therapeutic potential of IL-4 in severe malaria in *Plasmodium berghei* ANKA-infected mice. IL-4 treatment has led to reduced parasitemia and decreased mortality. Several mechanisms could be involved, including increased receptor expression for phagocytosis, high IgM synthesis, and decreased inflammatory processes in the brain (Wu *et al.,* 2021). However, it should be taken into consideration that, as in the case of the cytokines discussed above, the role of IL-4 depends on certain factors. It has been highlighted that dendritic cells release IL-4 early during malaria and in this case, IL-4 may contribute to the development of a severe form of malaria (Wu *et al.,* 2021). Elhussein *et al.* emphasized that IL-4 could be considered a risk factor for severe forms of malaria and that there is a positive correlation between IL-4 levels and parasitemia (Elhussein *et al.,* 2015).

Role of TGF-β in Malaria Pathogenesis

TGF-β has an anti-inflammatory effect by inhibiting Th1 cell differentiation and thus blocking the production of Th1-derived IFN-γ. TGF-β also inhibits Th2 cell differentiation, but this subset of cells does not appear to be involved in malaria (Drewry

and Harty, 2020). Several studies have shown a negative correlation between TGF-β levels and the severity of *P. falciparum* infection (Hanisch *et al.,* 2015). de Jong *et al.* (2020) have analysed the levels of several cytokines in a group of 15 volunteers before and during (at the day of treatment) a controlled malaria infection. They have reported that 9 subjects had low levels of TGF-β and high levels of IFN γ, IL-6, D dimer, and von Willebrand factor when compared to their respective baseline status. In contrast, in 6 subjects, the authors recorded elevated levels of TGF-β, with no changes in the other evaluated parameters, and among these patients, the symptoms were less severe in comparison to the other group (de Jong *et al.,* 2020). Keswani and Bhattacharyya (2013) have shown that the interplay between TGF-β and TNF-α is involved in splenocyte apoptosis in experimental brain malaria. Furthermore, they have observed that inhibition of TNF-α leads to a delay in splenocyte apoptosis, suggesting there probably is a competition between the two cytokines during infection. TGF-β and IL-6 modulate the function of several immune cells after a malaria infection, including dendritic cells, regulatory T cells, and T-helper cells (Th17) (Keswani *et al.,* 2016). According to a recent review, TGF-β is involved in the expansion of FoxP3 Tregs, an important mechanism to limit the inflammatory process in malaria (Drewry *et al.,* 2020).

2.13.5 Role of Other Cytokines in Pathogenesis of Malaria

de Menezes *et al.* (2019) have shown that neutrophils are an important source of IL-1α in the liver during malaria infection. IL-1α deficiency has been associated with lower levels of TNF-α, weight loss, and hypothermia but bears no significant effects on parasitaemia. The authors emphasized the potential role of IL-1α in the hepatic inflammatory process. Kisia *et al*, (2019) were the first to demonstrate the correlation between genotypes and

haplotypes of IL-7 (72194 T/C and -2440A/G) and inefficient erythropoiesis (Kisia *et al.,* 2019). A recent study showed that IL-12 and IL-18 play a central role in modulating the innate immune response in malaria. The synergistic effect of IL-12 and IL-18 seems to be involved in *γδ* T cell immunoregulation. The two cytokines are required for the up regulation of the inhibitory receptor TIM3, expressed by *γδ* T cells. The over-expression of TIM3 was associated with a decreased risk of clinical malaria (Schofield *et al.,* 2017). In a group of patients infected with *P. vivax*, research studies have shown that thrombocytopenia and low levels of IL-2 and IL-12 correlate with platelet distribution width (PDW) values, and thus concluded that these changes may represent an immune response to thrombocytopenia (Costa et al., 2020).

Aljarba *et al.* (2020) have reported that IL-22 polymorphisms in rs2227481 and rs2227483 are involved in the mounting of a protective immune response against *Plasmodium falciparum* infection. The authors also point out that the G allele of rs2227513 plays an important role in the increased production of IL-22. A recent study highlighted the role of IL-35 in malaria. Bello *et al*, (2020) have identified that IL-35 is overexpressed in the serum and tissues of *P. berghei*-infected mice. In addition, there was a positive correlation between the levels of IL-35 and parasitemia. IL-35 neutralization has been associated with beneficial effects on parasitemia, tissue histological changes, and even with a higher survival rate in *P. berghe*i-infected mice.

2.13.6 Summary of the Role of Cytokines in the Pathogenesis of Malaria

TNF alpha

Increase phagocytic uptake of parasites (Gbedande *et al*, 2020)

Elevated levels correlated with the severity of malaria (Boyle *et al*, 2015)

May be associated with cerebral malaria (Leao *et al*,2020)

Increase intracellular calcium levels and decrease the count of intracellular parasites (Cruz et al, 2015)

IFN gamma

Type 1 IFN signalling limits CD4+ T helper cell activity during the blood infection stage (Sebina and Haque, 2018)

Type 1 IFNs stimulate the release of proinflammatory cytokines (Sebina and Haque, 2018).

Participate in the control of infections (Hu *et al*, 2020)

Chronic high levels may lead to immune suppression (Hu *et al*, 2020).

IL-6

Participate in the control of infection (Sebina *et al*, 2017)

Promote the expression of ICOS in the T cells and activates the differentiation of B cells (Mbengue et al, 2016)

Is only involved in the early stages of infection (Mbengue et al, 2016)

Could be regarded as marker for severe malaria

Its level increases during the acute phase of malaria that persists through convalescence (Sebina *et al*, 2017)

IL-8

Correlates with severity of malaria (Mahanta *et al*, 2014)

Its levels increase during the acute phase of malaria that persist through convalescence (Mahanta *et al*, 2014)

IL-10

Inhibit protective immune response against secondary infection (Nakame *et al*, 2019).

Its inhibition is associated with increased CD4+ T cells activity, the release of IFN-γ and decreased parasitaemia (Boyle *et al*, 2017)

P. falciparum-specific IL-10 positive T cells (IFN- γTNF) correlate with the risk of clinical malaria (Boyle *et al*, 2017)

Elevated intracellular level in CD4+ T cells have a protective effect against P.falciparum and on haemoglobin level at delivery (Requena *et al*, 2019)

IL-4

Is an important regulator of Th2 responses (Wu *et al*, 2022)

Limit both the inflammatory process and Th1 responses (Wu et al, 2022)

Has a protective role in severe forms of malaria (Wu *et al*, 2022)

Could be considered a risk factor for several form of malaria (Wu *et al*, 2022)

TGF beta

Has an anti-inflammatory effect by inhibiting Th1 cell differentiation (Hanisch *et al*, 2015)

Negatively correlates with the severity of *P.falciparum* infection (Hanisch *et al*, 2015)

Modulates the function of several immune cells after a malaria infection, including dendritic cells, regulatory cells, and T-helper cells (Th17) (Keswani *et al*,2016)

Is involved in the expansion of FOX P3 Tregs (Drewry and Harty 2020).

2.14 Immunological Profile of Malaria

C-reactive protein (CRP): Is the first acute phase protein of inflammation and a known sensitive and prognostic marker in severe malaria. CRP is a helpful tool for predicting the severity of *P. falciparum* and it can also be used to differentiate complicated and uncomplicated malaria. CRP level in elevated in *P. falciparum* infection can be used diagnose malaria where clinical manifestation like fever may be absent but with high malaria parasitaemic level. Elevated CRP level is also reported in sepsis which causes severe inflammation in the body. Hyperparasitaemia does not necessarily have diagnostic significance in semi-immune individuals as they may tolerate parasitaemic burden without any clinical sign of the diseases or severe effect (Rovira-Vallbona *et al.,* 2012)

Cytokine Response: The initial response is a sharp burst of pre-inflammatory cytokines to restrict parasite growth and regulate the development of adaptive immunity. Interleukin 1-β (IL-β) and Interleukin 6 (IL-6) are well known inflammatory markers and play important role during induction of CRP expression level in human hepatocytes (Sebina *et al.,* 2017). The stimulation of CRP in the hepatocyte is controlled by the level of IL-6, a regulator that enhanced by IL-β

(Emmanuel *et al.,* 2021). The elevation CRP level during disease progression makes it widely used in inflammatory protein marker. The activation of platelet and complement pathway triggers CRP which results in mediation of effector pathway leading to complications. Therefore CRP measurement could be helpful in understanding the pathogenesis of severe malaria cases (Emmanuel *et al.*, 2021).

CRP as a Biomarker in Malaria

In Nigeria, elevated CRP level recognised as indicator of malaria parasitaemia especially *P. falciparum*. Research findings showed that there is strong positive correlation between CRP levels and malaria parasitaemia, indicating that CRP can be used as a biomarker for malaria diagnosis. High serum level of CRP indicates severe malaria in most cases. Hence, CRP testing is a valuable diagnostic tool for early detection and monitoring of malaria severity (Emmanuel *et al*, 2021).

CRP as a Biomarker

CRP is a protein produced by the liver in response to inflammation, and its level is elevated during infection including malaria (Wilairatana *et al*, 2021).

Correlation between CRP Level and Malaria Severity: Research finding indicate that CRP levels are significantly elevated in patients with malaria and higher serum associated with more severe cases and increased risk of complications (Abdelwahab *et al*, 2024).

CRP as a Diagnostic and Prognostic Tool: In differential diagnosis, CRP can be used to differential between malaria and other febrile illness. It is more pronounced in severe malaria than uncomplicated malaria. As such it can be used as a maker for severe malaria (Emmanuel *et al*, 2019).

Studies in Nigeria: Surveillance studies carried out in Nigeria revealed the role of CRP in malaria especially in Children. The findings shows that CRP levels can be used to differentiate between malaria and febrile illness and also predicting the severity of malaria (Emmanuel *et al*, 2019)

2.14.1 CRP Assay

C-reactive protein tests for malaria primarily focus on assessing the severity of the infection and distinguishing it from other fevers.

There are two main types of CRP tests used:

Quantitative Test: This test provides numerical value of CRP level (5-200 mg/L) and requires specialized equipment.

Semi-quantitative: These tests offer a range of CRP levels (e.g., positive/negative, or positive/semi-quantitative) and are often used at point-of-care (Emmanuel *et al*, 2019)

2.14.2 Cytokine Assay

The assay of various cytokines in malaria infection is primarily carried out to assess if there is inflammatory response and serum elevation of the parameters were involved. This assay uses Enzyme-linked immunosorbent assay (ELISA) technique, a

quantitative test that measures the concentration of cytokines in a given blood sample (Emmanuel *et al*, 2019)

2.14.3 Plasmodium *falciparum* Species

Plasmodium falciparum is the deadliest malaria parasite species and the most prevalent in Africa. Its genetic diversity leads to varied interactions with hosts, complicating treatment and control efforts (WHO, 2021).

The malaria parasite name, falciparum comes from the characteristic sickle shape of the gametocytes of this species (*falx*-sickle, *parere*-to bring forth). This is the most highly pathogenic of all the plasmodia and hence the name malignant tertian or pernicious malaria for its infection (Paniker, 2020) The disease has a high rate of complications and often highly fatal if untreated. The species is responsible for almost all deaths caused by malaria and is deeply entrenched in tropical Africa, parts of Asia, central and South America. It is limited to the tropical and subtropical regions because at low climatic temperatures below 20°C, the development of mosquito is greatly retarded (Paniker, 2020). This species is of the great public health importance due to its increasing resistance to antimalarial drugs and its spread to new areas. In India and Africa, it has been spreading widely, causing large epidemics in some places (Isaacs *et al.,* 2012).

The sporozoites have sickle-shape and the tissue phase consists of only a single cycle of pre-erythrocytic schizogony, no hypnozoites occur. The mature liver schizont releases about 30,000 merozoites which attack both young as well as mature erythrocytes and so the population of red cells affected is very large and the infected erythrocytes present a abnormal colouration (Bancells *et al.,* 2019).

In the early stage, the ring form in the erythrocyte is very fragile and tiny, measuring only a sixth of the red cell diameter. Rings are often seen attached to the margin of the red cell, the so-called *form applique* or *accole*. Binucleate rings are common appears and resemble stereo headphones in appearance. The rings may be seen within a single red blood cell and in the course of time, the rings become larger, about a third of the size of the erythrocyte and may have one or two grains of pigment in its cytoplasm (Vaughan *et al.,* 2012).

The subsequent stages of the asexual cycle late trophozoite, early and mature schizonts are not ordinarily seen in peripheral blood, except in very severe or pernicious malaria. The presence of *P.falciparum* schizonts in peripheral smears indicates a grave prognosis. The trophozoites usually disappear from peripheral circulation after about 24 hours. By then, a strain-specific high molecular weight antigen appears on the surface of the infected red cells, associated with knob-like projections on the erythrocyte membrane. Such red cells disappear from peripheral circulation and adhere to the walls of venules and capillaries in internal organs—brain, heart, kidney, lungs, spleen, intestine, bone marrow, placenta. This cytoadherence causes sequestration of infected red cells in, these sites and is responsible for many of the serious complications of falciparum malaria, such as cerebral malaria (Mok *et al.,* 2023).

The mature schizont is smaller size than in any other species and has between 8-24 merozoites. The erythrocytic schizogony takes about 48 hours or less, so that the periodicity of febrile paroxysms is 36 to 48 hours. Very high intensity of parasitisation is seen in falciparum malaria. In very severe infections the rate of

parasitised cells may even be up to 50 per cent. The infected erythrocytes are of normal size. They show a few (6-12) coarse brick-red dots which are called *Maurer's clefts*. Some red cells show basophilic stippling (Dharmadhikari *et al.,* 2013).

The gametogony begins after several generations of schizogony and are seen in circulation about 10 days after the first appearance of the ring stage. The early or immature gametocytes seldom appear in peripheral circulation, but the mature ones which are seen in peripheral smears are curved oblong in structures often described as crescentic, sickle, sausage or banana shaped. They are usually referred to as crescents. The male gametocytes are broad and sausage-shaped or kidney-shaped; with blunt rounded ends as compared to the female gametocytes which are thinner and more typically crescentic, with sharply rounded or pointed ends. The mature gametocyte is longer than the diameter of the red cell and so produces gross distortion and sometimes even apparent disappearance of the infected red cell. The red cell is often seen as a rim on the concave side of the gametocyte. The cytoplasm in the female gametocyte is deep blue, while in the male it is pale blue or pink. The nucleus is deep red and compact in the female, with the pigment granules closely aggregated around it, while in the male it is pink, large and diffuse, with the pigment granules scattered in the cytoplasm. *P. falciparum* crescents can survive in circulation for up to 60 days, much longer than in other *Plasmodium* species. Gametocytes are most numerous in the blood of young children, 9 months to 2 years old. They therefore serve as the most effective source of infection to mosquitoes (Ngwa *et al.,* 2016).

2.15. Life Cycle of Plasmodium falciparum

The name *falciparum* originates from the characteristic sickle shape of the gametocytes of this species (*falx*-sickle, *parere*-to bring forth). This species is the most highly pathogenic of all the plasmodia and hence the name malignant tertian or pernicious malaria for its infection. The disease is associated with high rate of complications and unless treated is often fatal. The species is responsible for almost all deaths caused by malaria and it is highly established in tropical Africa and some parts of Asia. It is also limited to the tropical and subtropical regions because favourable of temperatures below 20°C, its development in the mosquito is greatly retarded. These species are of the great public health importance due to its high prevalence, increasing resistance to antimalarial drugs and its spread to new areas. In tropical Africa, it has been spreading widely, causing large epidemics in some places (Bousema *et al.,* 2010).

The sporozoites are sickle-shaped and the tissue phase consists of only a single cycle of pre-erythrocytic schizogony, no hypnozoites occur. The mature liver schizont releases about 30,000 merozoite and they attack both young and mature erythrocytes and so the population of cells affected is very large give infected erythrocytes present a brassy colouration (Jennifer and Catherine, 2022).

The early ring form in the erythrocyte is very delicate and tiny, measuring only a sixth of the red cell diameter. Rings are often seen attached along the margin of the red cell, the so-called *form applique* or *accole*. Binucleate rings are common resembling stereo headphones in appearance. Several rings may be seen within a single erythrocyte. In course of time, the rings become larger, about a third of the size of the red cell and may have one or two grains of pigment in its

cytoplasm (Scott *et al.,* 2012).

The subsequent stages of the asexual cycle late trophozoite, early and mature schizonts are not ordinarily seen in peripheral blood, except in very severe or pernicious malaria. The presence of *P.falciparum* schizonts in peripheral smears indicates a grave prognosis. The trophozoites usually disappear from peripheral circulation after about 24 hours. By then, a strain-specific high molecular weight antigen appears on the surface of the infected red cells, associated with knob-like projections on the erythrocyte membrane. Such red cells disappear from peripheral circulation and adhere to the walls of venules and capillaries in internal organs brain, heart, kidney, lungs, spleen, intestine, bone marrow, placenta. This cytoadherence causes sequestration of infected red cells in, these sites and is responsible for many of the serious complications of falciparum malaria, such as cerebral malaria (Akide, 2022).

The mature schizont is smaller than in any other species and has 8 to 24 (usually 16) merozoites. The erythrocytic schizogony takes about 48 hours or less, so that the periodicity of febrile paroxysms is 36 to 48 hours. Very high intensity of parasitisation is seen in falciparum malaria. In very severe infections the rate of parasitised cells may even be up to 50 per cent. The infected erythrocytes are of normal size. They show a few (6-12) coarse brick-red dots which are called *Maurer's clefts*. Some red cells show basophilic stippling (Talleh *et al.,* 2014).

Gametogony begins after several generations of schizogony. Gametocytes are seen in circulation about 10 days after the ring stage first appears. The early gametocytes seldom appear in peripheral circulation. The mature gametocytes which are seen in peripheral smears are curved oblong structures variously described as crescentic, sickle,

sausage or banana shaped. They are usually referred to as crescents. The male gametocytes are broad and sausage-shaped or kidney-shaped; with blunt rounded ends as compared to the female gametocytes which are thinner and more typically crescentic, with sharply rounded or pointed ends. The mature gametocyte is longer than the diameter of the red cell and so produces gross distortion and sometimes even apparent disappearance of the infected red cell. The red cell is often seen as a rim on the concave side of the gametocyte. The cytoplasm in the female gametocyte after staining appear deep blue, while in the male it is pale blue or pink. The nucleus stains deep red and compact in the female, with the pigment granules closely aggregated around it, while in the male it is pink, large and diffuse, with the pigmented granules scattered in the cytoplasm. *Falciparum* gametocyte which is crescent in shape can survive in circulation for up to 60 days, much longer than in other *Plasmodium* species. Gametocytes are most abundant in the blood of young children, 9 months to 2 years old they therefore serve as the most effective source of infection to mosquitoes (Panika, 2022*)*

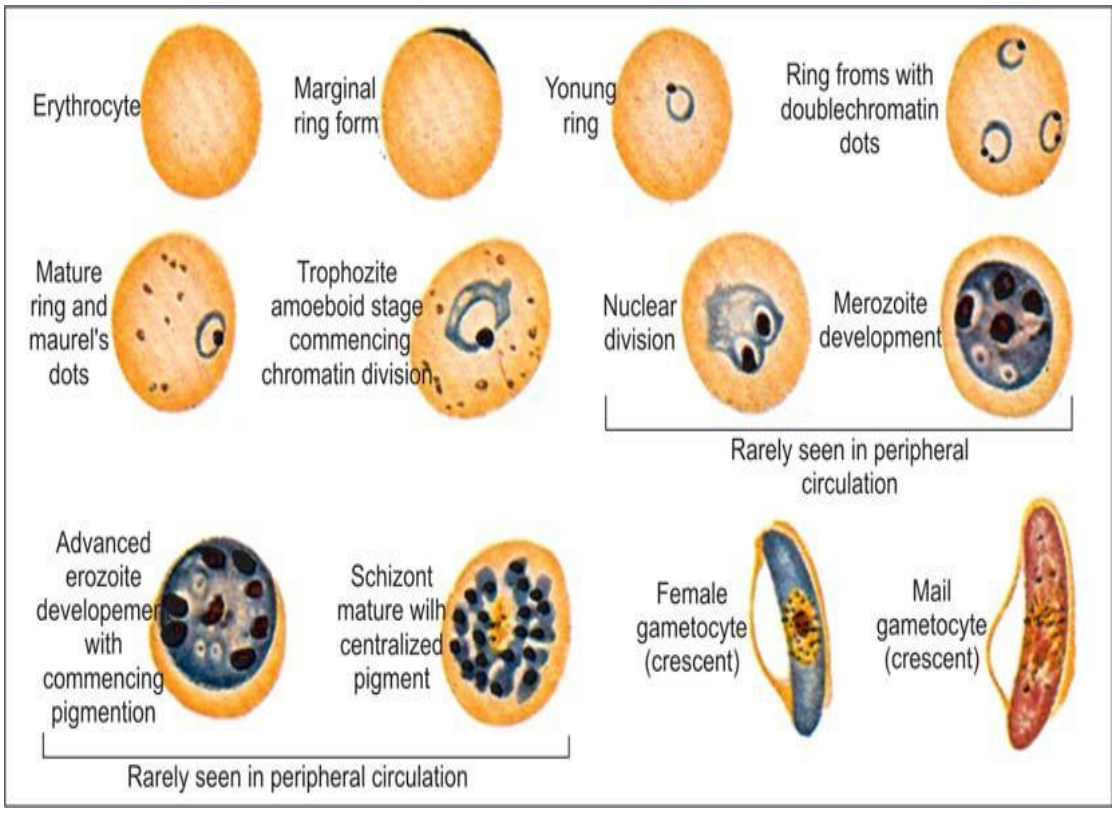

Figure 2.6: *Plasmodium falciparum* (Giemsa stain, magn x 2000)

Source: Panika, 2020

2.15.1 Strains of Plasmodium Falciparum

Plasmodium falciparum responsible for the most severe form of malaria exist in numerous strains isolated and collected from different geographical regions of the word. There are different strains of *P. falciparum* are associated with varying levels of drug resistance and geographical distribution (Talleh *et al.,* 2014).

2.15.2 Features of Strains of P. falciparum

Genetic Diversity: These strains display genetic diversity influencing the severity of malaria symptoms and the existence of within-host competition among different strains (Amabua-Ngwa *et al*, 2018).

Genome Structure: The genomic sequence has revealed variation in genome size among different P. falciparum strains with some strains being cleaved by several hundred thousand of base pairs smaller than others (Amabua-Ngwa *et al*, 2018).

The key strains include:

***P. falciparum* 3D7:** A strain commonly used in research, cloned from NF54, and often used as reference material genetics studies. It originates from Europe and Asia and is resistant to chloroquine and pyrimethamine.

***P. falciparum* NF54:** Is a progenitor strain of 3D7, collected from Netherland

***P. falciparum* K1:** This strain, originating from Thailand, is often used in laboratory experiments focusing on the parasite's ring stage.

***P. falciparum* HB3:** It is a commonly used strain, cloned from patients in Honduras; it is known for resistance to antimalarial drugs like chloroquine.

***P. falciparum* Dd2:** Commonly found in the Indo-China region, this strain is resistant to pyrimethamine.

***P. falciparum* D6:** Originating from Sierra Leone, it is prevalent in Africa.

***P. falciparum* Full-length cDNA Bank (FCB):** A strain valuable for functional genomic studies.

***P. falciparum* IT4:** A strain from Vietnam resistant to chloroquine and pyrimethamine.

***P. falciparum* CAMP:** A strain from Mali, used in studies of malaria pathogenesis and immunity (CDC 2020, Mohamed *et al.,* 2021 and Pham *et al.,* 2021)

The study of these varieties of different subtypes helps researchers to develop more effective treatment and vaccine development for malaria

2.16 *Plasmodium falciparum* Histidine Rich Protein 2 Gene

Plasmodium falciparum Histidine-Rich Protein 2 (HRP2) is a protein produced by the parasite *Plasmodium falciparum*, which causes malaria. PFHRP2 is a 25 kDa protein composed of repeating units of histidine and alanine amino acids, which gives it a high affinity for binding to haematin, a by-product of haemoglobin degradation. This binding ability helps the parasite evade the host's immune system (Amoah *et al.,* 2016).

The detection of HRP2 in blood samples is a widely used diagnostic tool for malaria, particularly in resource-limited settings where microscopic examination of blood smears is not feasible (WHO, 2021). However, HRP2-based RDTs have limitations, such as the potential for false-negative results due to genetic deletions or mutations in the HRP2 gene (WHO, 2022).

2.16.1 *Plasmodium falciparum* Histidine-Rich Protein 2 Gene Deletion

PfHRP2 is the target antigen for most RDTs based test kits. Gene deletions or mutations of the *pfHRP2* gene in samples infected with *P. falciparum* can cause false-negative results (Gupta *et al.*, 2017). If PfHRP2 gene is deleted, the malaria parasite does not produce Pfhrp2 protein, and the RDT based kit will incorrectly indicate absence of malaria parasite. This can have adverse consequence on malaria control effort as untreated malaria can present high morbidity and mortality (Fuenta et al, 2021). In 2010, Gamboa *et al* reported the first confirmed identification *Plasmodium falciparum* parasite isolate with PfHRP2 and PfHRP3 gene deletion, these parasites expressed neither PfHRP2 nor PfHRP3 genes, and were identified in Amazon River basin of Peru. This phenomenon threatens malaria control strategies, as the resulting misdiagnoses could affect malaria diagnosis, management, and treatment outcomes. However, the failure to detect and treat false-negative infection increases the risk that people in a given community can contribute to onward infection through mosquito's bite transmission. The majority of commercial RDT currently available detect *P. falciparum* only and expressed in blood during ring stage (Bharti *et al,* 2016 and Prosser *et al.,* 2021)

2.16.2 Effect of Pfhrp2 Gene Deletions on RDTs

The majority of RDTs rely on detecting Pfhrp2 antigen, a specific protein encoded by the pfHRP2 gene. False-negative results may be due to factors such as low parasite density, but gene deletions remain the primary cause (WHO, 2018). The spread of pfHRP2 and pfHRP3 gene deletions may be influenced by factors like regional malaria prevalence, treatment-seeking behaviours, age, and symptom type (Fuenta *et al*., 2021). A PfHRP2 gene deletion causes false RDT-negative and false-negative result is gotten from that sample which is negative by RDT but positive by another diagnostic method especially by molecular diagnosis (Bharti *et al., 2016*). If PfHRP2 gene is deleted, the parasite does not produce the

Hrp2 protein, and the RDT will incorrectly indicate the absence of malaria. This can have serious consequence for malaria treatment and control efforts, as untreated malaria can be deadly (Fuenta *et al*., 2021)

2.16.3 Prevalence of Pfhrp 2 Gene Deletions

The prevalence of pfHRP2 deletion varies geographically, with the highest rates observed in South and Central America, followed by Africa and Asia (Koita *et al.,* 2012). For pfHRP3, deletion rates are similar, while double deletions of both Pfhrp2and Pfhrp3 genes range from 0% to 25% in South and Central America, 0% to 62% in Africa, and up to 4% in Asia. In African countries; Mali (5%), Ghana (29%), Equatorial Guinea (5.3%), Cameroun (6.3%) Democratic Republic of Congo (DRC) (6.4%), Eritrea (62%) and Rwanda (9.2%) The World Health Organization (WHO) recommends changing RDT kits if the prevalence of *pfhrp2* deletions exceeds 5%, as this significantly impacts test accuracy and public health strategies (WHO, 2016).

2.16.4 Limitation of Pfhrp2 Based RDTs

The major limitations of the use of RDT are false positive result because PfHRP2 protein persists in the blood several days after an infection has been cleared (Humar *et al,* 1997). And false negative that can be due to PfHRP2 gene deletions as it has been observed for pfhrp2 in field isolate reported in Africa (WHO*,* 2016).

2.17 Laboratory Diagnoses of Malaria

The World Health Organisation recommends prompt parasitic-based and accurate laboratory diagnosis of malaria in all cases of malaria in all cases. This is critical for effective treatment and prevention of community spread. The chosen diagnostic method must be reliable and suited for medical emergencies (Calderado *et al.,* 2024).

2.17.1 Microscopic Diagnosis

Microscopic examination of blood films is a gold standard for malaria diagnosis. The thick blood film is used for parasite detection, while thin blood films are used for species identification. Accurate results depend on high-quality reagents, equipment, and skilled microscopist. However, negative blood films cannot entirely rule out malaria, and slide preparation takes 30–45 minutes on average (CDC, 2020).

Malaria Microscopic Standard Operating Procedure

The following are standard operating procedure for a well-prepared blood firm for microscopic examination of malaria parasite.

Cleaning and storing microscopic slide

Preparation of Giemsa stock solution

Preparation of water buffered to pH 7.2

Preparation of water buffered to pH 7.2 with buffer tablets

Quality control of Giemsa stock solution and buffered……

Preparation of Giemsa working solution

Collecting of finger-prick blood and preparation of thick and thin blood films

Collection of blood by venepuncture and preparation of blood films from venous blood

Giemsa staining of blood films

Labelling malaria blood films

Recording and reporting microscopic results

Microscopic examination of thick and thin blood for identification of malaria parameters

Management of results generated from malaria diagnostic test (WHO, 2021)

2.17.3 Antigen Detection

Rapid diagnostic test is based on antigen detection, provide rapid results and are particularly useful in areas without reliable microscopic diagnosis. The U.S. FDA approved RDT use in 2007 for clinical laboratories, with the condition that positive results be confirmed microscopically to determine species and quantify parasite load (FDA, 2007). RDTs have revolutionized malaria diagnosis by delivering results in minutes, making them invaluable in emergency and resource-limited settings (FDA, 2007).

There are several types of malaria antigens detection in diagnosis these including:

i. Histidine-Rich Protein 2/3 (HRP2/3): A protein produced by *Plasmodium falciparum*, used in rapid diagnostic tests (RDTs) and enzyme-linked immunosorbent assays (ELISAs).
ii. *Plasmodium falciparum* Lactate Dehydrogenase (pFLDH): An enzyme produced by *P. falciparum*, used in RDTs and ELISAs.
iii. *Plasmodium vivax* Lactate Dehydrogenase (pVLDH): An enzyme produced by P. vivax, used in RDTs and ELISAs.
iv. Aldolase: An enzyme produced by Plasmodium species, used in RDTs and ELISAs.
v. Circumsporozoite Protein (CSP): A protein produced by *Plasmodium* species, used in ELISAs and indirect fluorescent antibody tests (IFATs).

vi. Merozoite Surface Protein 1 (MSP1): A protein produced by *Plasmodium* species, used in ELISAs, IFATs and PCR.

vii. Apical Membrane Antigen 1 (AMA1): A protein produced by *Plasmodium* species, used in ELISAs and IFATs.

viii. Glutamate Dehydrogenase (GDH): An enzyme produced by *Plasmodium* species, used in RDTs and ELISAs.

ix. *Plasmodium falciparum* Glutamate Dehydrogenase (FfGDH): An enzyme produced by P. falciparum, used in RDTs and ELISAs and PCR.

x. Pan-Plasmodia Antigen: A combination of antigens from multiple Plasmodium species, used in RDTs and ELISAs to detect malaria infections regardless of the species (Ugah *et al*, 2017).

These antigens are used in various diagnostic tests, including RDTs, enzyme linked immunosorbent assays (ELIZAs), immunofluorescence assay techniques (IFATs), Western blots and PCR, to detect malaria infections and identify the specific *Plasmodium* species (CDC,2020). The majority of commercial RDT kits currently available, only *Plasmodium falciparum* that is expressed in the ring stage (Bharti *et al.,* 2017) In the absence of a well-trained microscopist for microscopic diagnosis of malaria in many especially remote areas, WHO recommends the use of RDTs as a good alternative for malaria diagnosis (Ugah *et al.,* 2017; Kim *et al.,* 2008)

2.18 Molecular Technique for Malaria Diagnosis

This technique involves detecting the parasite nucleic acids using polymerase chain reaction (PCR). This is the gold standard, and it is recommended that all RDTs are followed-up with microscopy or PCR to confirm the results, and if positive, confirm the species and quantify the proportion of red blood cells that are infected. Polymerase Chain Reaction (PCR):

Amplifies specific DNA sequences from malaria parasites, allowing for sensitive detection (CDC, 2022).

2.18.1 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a versatile powerful molecular biology technique used to amplify specific DNA or RNA sequences, making it easier to detect and analyze genetic material. PCR is widely used in medical diagnostics, forensic science, genetic research, and infectious disease surveillance.

The principle is based on enzymatic replication of the nucleic acids. The double-stranded DNA of interest is denatured and separated into two individual strands.

Each strand of DNA is then allowed to hybridize with a primer.

The primer-template duplex is used for DNA synthesis by the enzyme DNA polymerase. The above is repeated until the process is stopped (Mullis and Faloona, 1987)

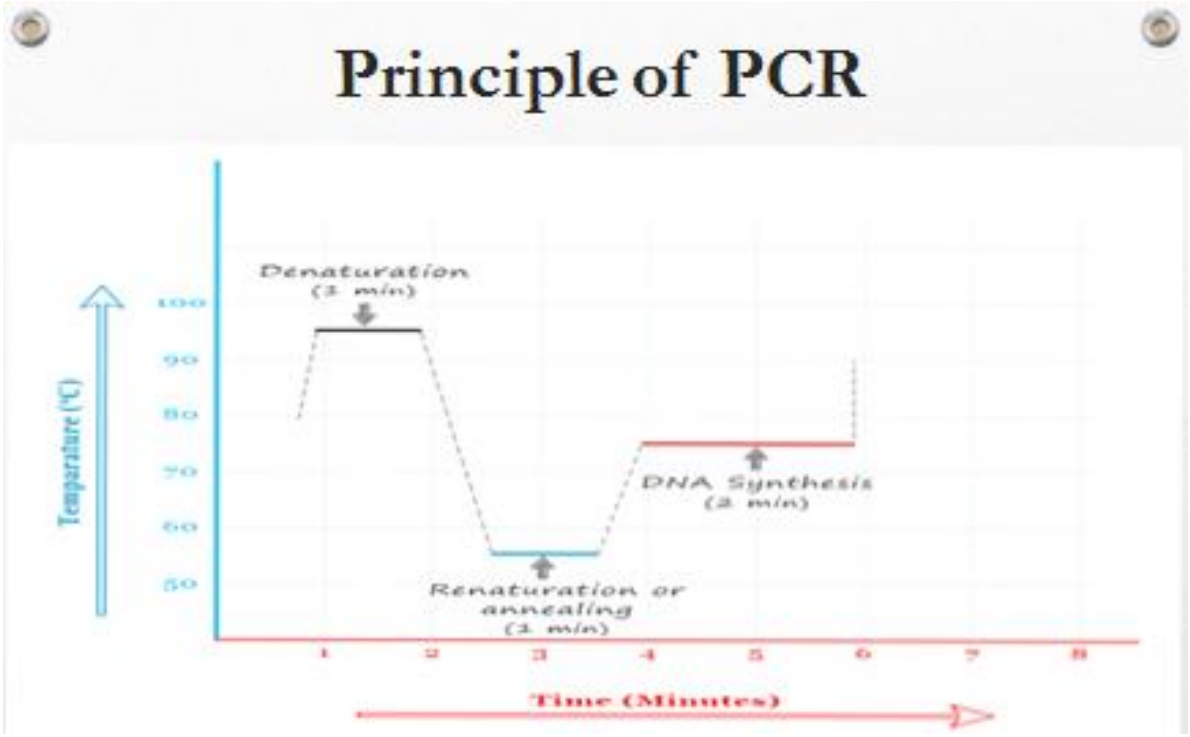

Figure 2.7: Principle of PCR

Source: PCR World

Mackay, (2004).

2.18.2 Procedure of PCR

The actual technology of PCR involves repeated cycles to amplify target DNA. Each cycle in the PCR technique has three steps,

i. Denaturation
ii. Renaturation or annealing
iii. Elongation

These PCR steps are related to temperature and time, therefore, each cycle in PCR takes about 3−5 minutes.

On raising the temperature to about 95 ℃ for about 0.5 to 2 minutes, the DNA gets denatured, and two strands are separated by breaking the hydrogen bond between the two strands of DNA. Such types of single strands now act as a template for the production of new DNA strands.

The temperature in the denaturation of the polymerase chain reaction is provided for a longer time to ensure the separation of the two strands. In normal practice, the PCR is carried out in an automatic machine (Mullis and Faloona, 1987).

- **Denaturation:** On raising the temperature to about 95 ℃ for about 0.5 to 2 minutes, the DNA gets denatured, and two strands are separated by breaking the hydrogen bond between the two strands of DNA. Such types of single strands now act as a template for the production of new DNA strands.

The temperature in the denaturation of the polymerase chain reaction should be provided for a longer time to ensure the separation of the two strands.

- **Annealing:** When the temperature of the mixture is slowly cooled to about 55 ℃, the primer bases pair with the complimentary reagins flanking target the DNA strands. Such a process is called renaturation or annealing. In this step, the primers bind to

their complementary sequences on the template DNA. A high concentration of premier ensures annealing between each DNA strand and the primer rather than the two stands of DNA. The two separated strands in the polymerase chain reaction run in the opposite direction. Therefore, the two primers such as forward primer and reverse primer is used in renaturation or annealing.

- **Elongation:** At the elongation step, the temperature is raised to 72-80℃ and initiation of DNA extension occurs at the 3′ − hydroxyl end of each primer. The premiers are extended by joining the bases complementary to DNA strands by the Taq polymerase enzyme. Therefore, the synthetic process in PCR is quite comparable to the DNA replication of the leading strand.

Almost all PCR machines contain a heat-stable DNA polymerase, such as Taq polymerase. It is an enzyme that is isolated from the thermophilic bacterium Thermus aquaticus.

The enzyme taq polymerase can tolerate very high temperatures and adds DNA bases to the single strand. Therefore, a double-stranded DNA molecule is obtained during elongat6ion (Nolan *et al.,* 2006)

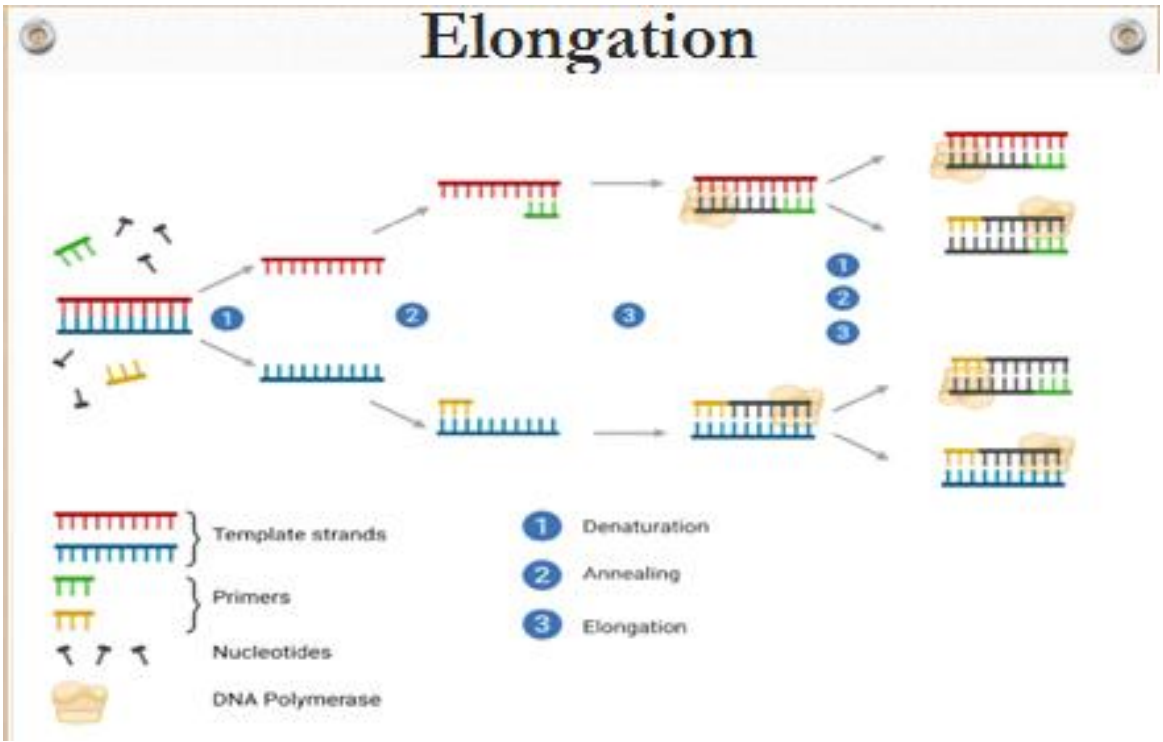

Figure 2.8: Different Stages of PCR

Source: BioModel

Mackay, (2004).

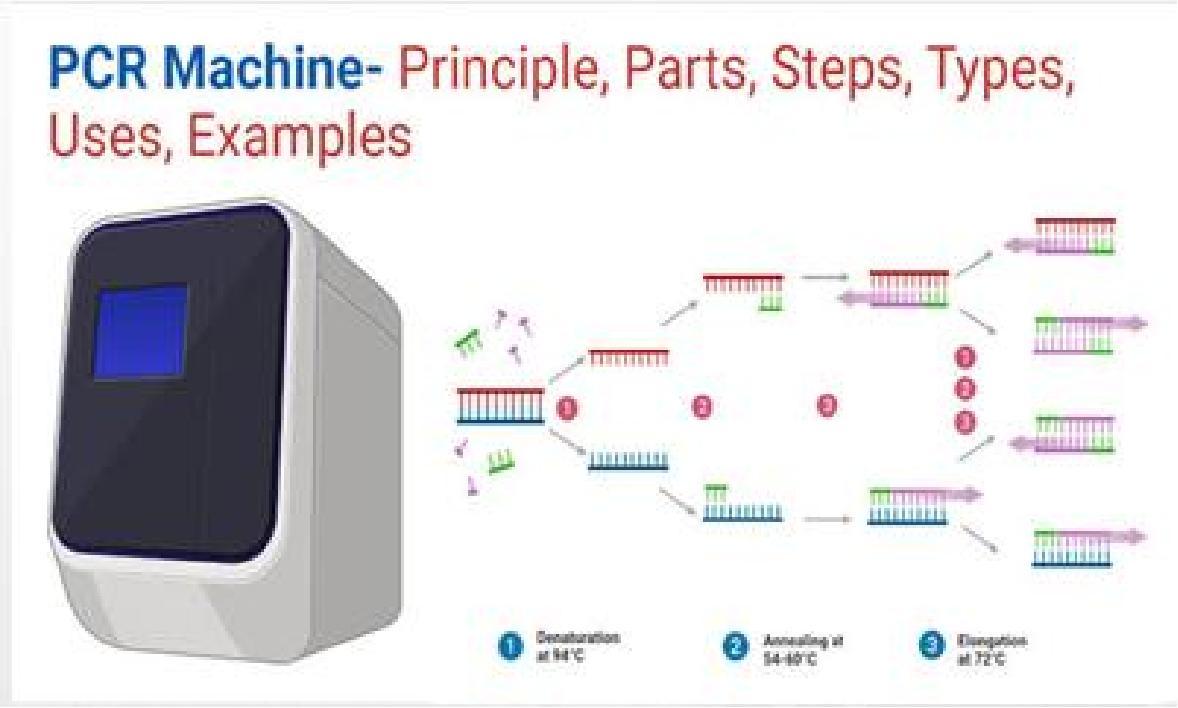

Figure 2.9: PCR Machine with Working Principles

Source: PCR Worlds

Mackay, (2004).

2.19 Types of molecular techniques (PCR) for detecting malaria parasites

Conventional PCR: It is most commonly used PCR technique used to amplify specific DNA sequence. The process involves repeated cycle of denaturation, annealing and elongation. The method can be complemented with real-time PCR. It can be used for DNA detection, amplification and diagnostic testing.

Real-Time PCR (qPCR): Monitors PCR amplification in real-time, enabling rapid detection and quantification. This method contains a thermal cycle that can illuminate each nucleic acid sample with a beam of light and one specific wavelength and detect by fluorescence emitter excited by the fluorophore (Mackay, 2004)

Loop-Mediated Isothermal Amplification (LAMP): A simple, rapid, low cost with high sensitivity and specificity (Ta *et al.,* 2011 and Gamboa *et al.,* 2017).

Inverse PCR: Inverse polymerase chain reaction as the name implies is only used for DNA amplification in one sequence. It is a PCR variant that is carried out even if only one sequence is available (Mullis and Faloona, 1987).

Reverse Transcriptase PCR: Is a polymerase chain reaction that is used to detect and amplify RNA by first converting it into complementary DNA (cDNA) using reverse transcription.

Multiplex PCR: This type polymerase chain reaction is a method that is used for the amplification of multiple samples within a single PCR analysis; it allows simultaneous amplification of multiple target DNA sequence in a single reaction. It uses multiple primers set in a single PCR mixture and amplifies many different DNA sequence simultaneously. The multiple primers sets are designed to work at the same annealing temperature during PCR experiment. M-PCR detects multiple malaria species or genes in a single reaction (Nolan *et al,* 2006)

Nest PCR: The nested polymerase chain reaction (N-PCR) is a method usually designed to improve sensitivity and specificity of DNA amplification or testing. It involves two separate PCR reactions; the first reaction is used as the second template for the second PCR, using inner primers that are located within the sequence amplified by the outer primer. There are two amplification reactions that take place and each of these uses a different set of primers. Hence it may reduce the non-binding of the product due to the amplification of unexpected primer binding sites.

Nested multiplex PCR: This is a combination of nested and multiplex PCR technique to achieve the desire reaction. The method amplifies multiple DNA sequence simultaneously. It uses two set of primers and two rounds of PCR to increase sensitivity and specificity. This technique is useful for detecting small quantities of DNA and RNA

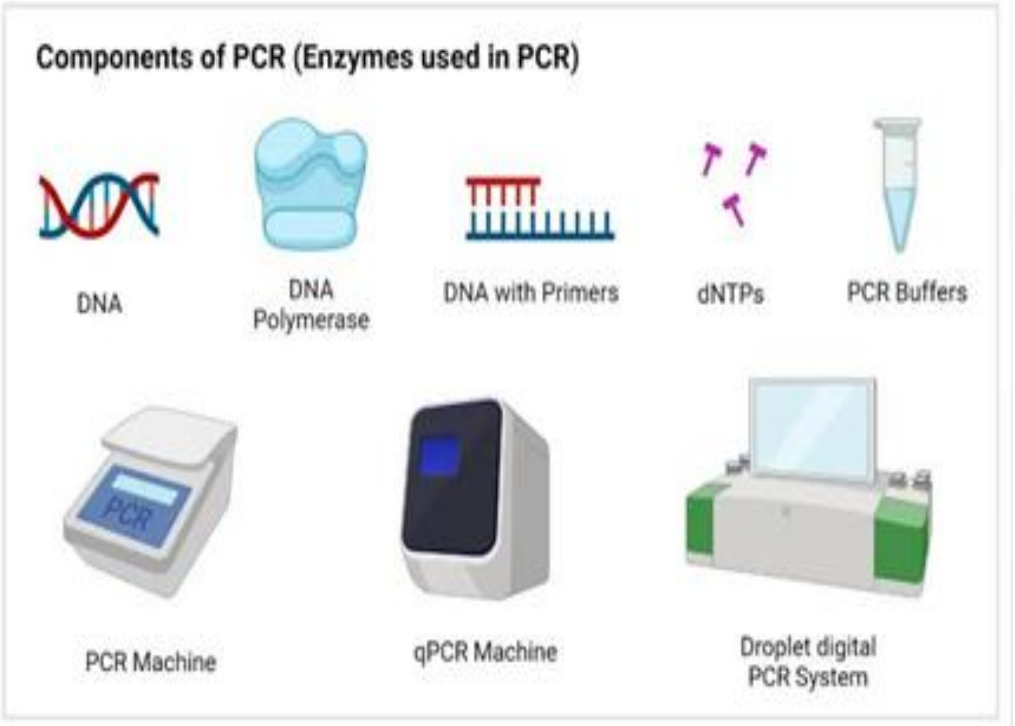

Figure 2.10: Different Component and Types of PCR Machine

Source: BioModel

Mackay, (2004)

2.20 Nested Multiplex PCR Technique for Malaria Diagnosis

First Round of PCR (outer primer): These primers are designed to bind outer region flanking target DNA sequence amplifying a large fragment.

Second Round of PCR (inner primer): These primers used in the second round of PCR are designed to bind within the amplified fragments from the first round, amplifying a specific target.

Simultaneous PCR Amplification: The multiplex PCR allows for concurrent amplification of various targets in single reaction, increasing efficiency and reducing cost (Chaianantakul *et al.,* 2022).

2.20.1 Advantages of Nested Multiplex PCR

Increased Sensitivity: Low-copy target are detected due to two rounds of amplification which decreases non-specific target amplification.

Simultaneous Deletion: NM-PCR allows for detection multiple targets in a single reaction, saving time and resources.

2.20.2 Application of Nested Multiplex PCR

Molecular Diagnostics: The Nested multiplex polymerase chain reaction can be used to identify specific DNA and RNA sequence in a given sample.

Medical Diagnostics: The NM-PCR is for medical diagnosis of bacteria, fungi, viruses and other pathogenic organism.

Genetic Research: NM-PCR is employed in research for the study of gene expression and identifying pathogens (Chaianantakul *et al.,* 2022).

2.21 Signs and Symptoms of Malaria

The first symptoms are fever, headache and chills- may be mild and difficult to recognize as malaria. If untreated within 24 hours, *P. falciparum* can progress to severe illness, often leading to death. Children with severe malaria frequently develops one or more of the following symptoms; severe anaemia, respiratory distress in relation to metabolic acidosis or cerebral malaria. In adult multi-organ failure is frequent, and in malaria endemic areas, people may develop partial immunity, allowing asymptomatic infection to occur (Cheesbrough, 2016).

2..21.1 Prevention and Treatment

2.21.2 Prevention of Malaria

Malaria infection can be prevented can be prevented through the following:

Avoiding mosquito bite by use of mosquito nets when sleeping in malaria endemic areas

Use of mosquito repellents'.

Wearing of protective clothing's

Use of window screens (WHO, 2022)

2.21.2.1 Vector Control

Vector control strategies aim to eliminate mosquitoes, which spread the malaria parasite through bites during blood meals.

These control methods include:

Insecticide-treated nets (ITNs) and Indoor Residual Sprayed Nets (IRS): These tools reduce mosquito-human contact and have been shown to significantly lower malaria transmission rates (World Health Organization (WHO, 2021).

Drainage systems: Proper drainage prevents stagnant water and waste accumulation, which serve as breeding grounds for mosquitoes (Centre for Disease Control and Prevention (CDC, 2020).

Chemoprophylaxis: Preventive treatment is recommended for travellers to malaria-endemic areas, as well as vulnerable groups such as pregnant women and children. Preventive chemotherapy includes Post-Discharge Malaria Chemotherapy (PDMC), Perennial Malaria Chemotherapy (PMC), Intermittent Preventive Treatment in Pregnancy (IPTp), and for school-age children (IPTsc) (WHO, 2021).

Chemotherapy: Treatment with antimalarial drugs is used to manage infected patients, providing a cost-effective way to complement broader malaria control strategies (CDC, 2020).

Vaccines: The RTS,S/AS01 malaria vaccine has been recommended by WHO for widespread use among children in regions with moderate to high *Plasmodium falciparum* transmission, marking a significant step in malaria prevention (WHO, 2021).

2.21.3 Treatment of Malaria

Early diagnosis and treatment are critical in alleviating malaria, preventing morbidity, mortality, and transmission. As recommended by the WHO, all suspected cases of malaria must be confirmed using parasite-based diagnostic testing, either through microscopy or rapid diagnostic tests (RDTs). Accurate testing ensures timely and effective treatment, reducing the disease burden (WHO, 2021; CDC, 2020).

Antimalaria treatment should completely destroy all asexual forms of the parasite in order to cure the clinical illness, eliminate sporozoites and exoerythrocytic forms to prevent relapse and kill gametocytes to block transmission to the vector mosquito. No

single drug satisfies and eliminates all parasitic forms, therefore combinations of drugs are necessary. The main strategy of treatment is to cure the clinical disease with blood schizonticidal drugs such as chloroquine (600 mg start, 300 mg after 6 hours, 300 mg daily for the next two days). Single dose regimens have also been advocated but may not always be adequate. Chloroquine does not destroy exoerythrocytic parasites and so in *P. vivax* and *P. ovale* infections, a tissue schizonticidal drug such as primaquine (15 mg daily for 5 days) should be administered. Primaquine is also active against gametocytes.

A major and serious problem may arise due to the development and spread of drug resistance in *P.falciparum*, which is now widespread in Africa, South America and South East Asia, including India. A combination of sulphadoxine and pyrimethamine (fansidar) or mefloquine is useful in such cases. In severe drug resistant falciparum malaria, intra- venous quinine may be lifesaving Panieka, 2018.

The traditional Chinese medicine Qinghaosu (from the shrub *Artemesia anhua*) and its derivatives artemether, artesunate and others have been found to be highly effective and safe antimalarial drugs (Ashley *et al*, 2014 and Panieka, 2018).

2.23 Immunological Markers and Parameters for Diagnosis of *P. falciparum*

The immunological markers and parameters is based on antigen antibody reaction and this has played significant role in the diagnosis of *P. falciparum* and other malaria species (Bharti *et al*, 2017). The immunological markers for *P. falciparum* include;

Pfhrp2

Pfhrp3

Circumsporozoite Protein (CSP)

Merozoite Surface Protein (MSP1)

Apical Membrane Antigen (AMA 1)

Pan-Plasmodia Antigen. (Klin *et al*, 2008)

CHAPTER THREE
MATERIALS AND METHOD

3.1 Study Area

The study was carried out in Kaduna, Kaduna State, Northwest zone, Nigeria. Kaduna has a tropical continental climate with two different seasons:

3.1.1 Climate of the Study Area

Wet season: The rainy seasons starts from March/April and ends September/ October (6 to 7 Months). The mean annual rainfall is between 1,323 and 1,525 mm. The peak rainfall in August has mean increase of 303.32 mm (NiMet, 2021)

Dry season: This commences from the end of October/November to March of the following year (5 to 6 months). The mean temperature of 21^0C is the coolest month (December to January while the mean temperature of the hottest months is 31^0C (March to April) (NiMet, 2021).

3.1.2 Kaduna

Kaduna is the capital of Kaduna State, the seat of the state government and the former political capital of Northern Nigeria. It is located in the north-west region of Nigeria on the either side of Kaduna River. It has an area of 431 km2 (166 square meter) with a population of 760,084 (2006 Census). It has a population density of 1,760 km2 (4,570/sqmi).

3.1.3 Ethnic Groups

Major ethnics groups in Kaduna metropolis are Hausa, Gbagyi, Adarra, Atyap- Bajju, Ham, Yoruba, Fulani, Kanuri, Margi, Nupe and Igbo. Rapid urbanization has increased the population estimated as at 2024 to be 1.2 million.

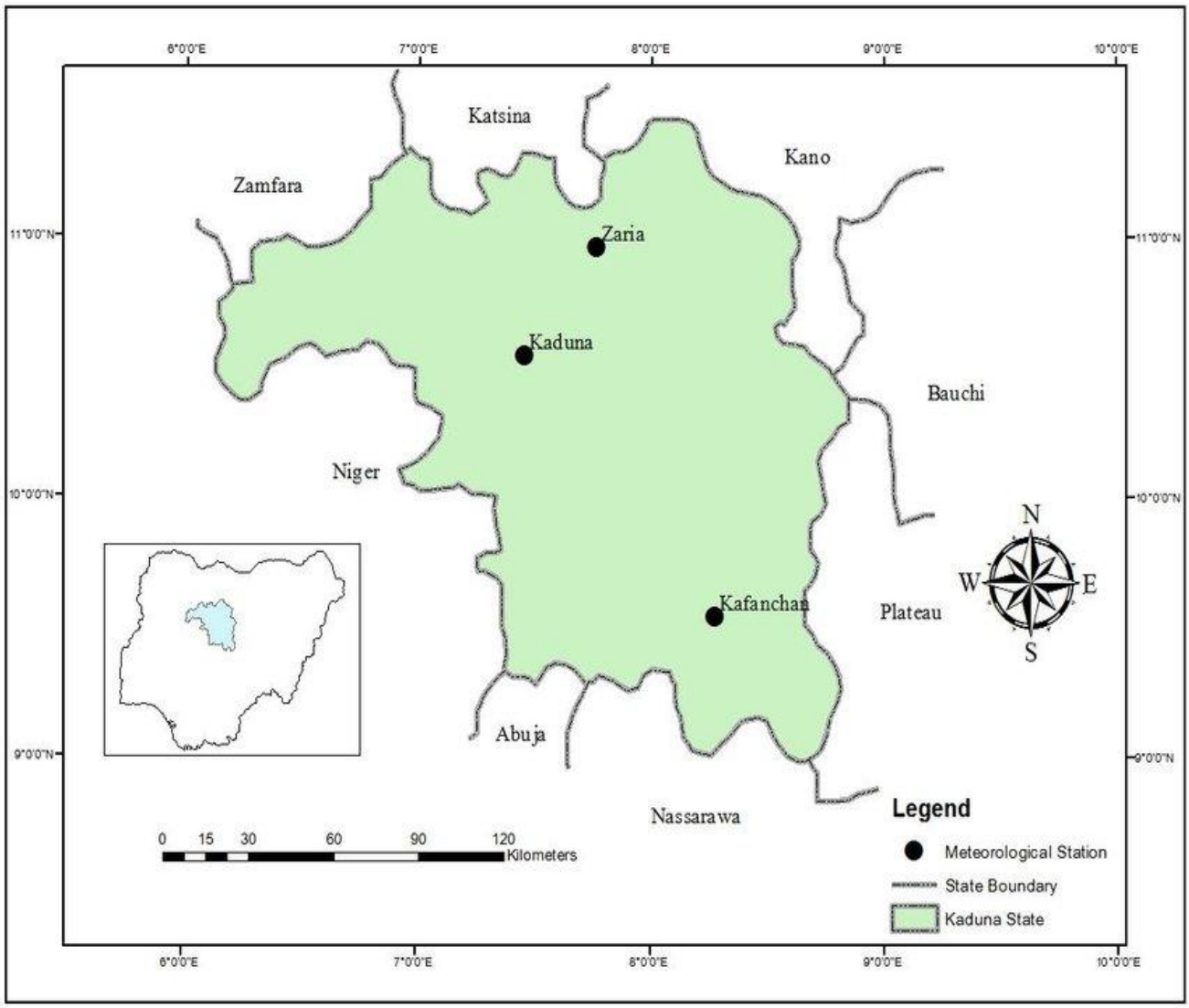

Figure 3.1.: Map of Kaduna Depicting Climatic Condition

Source: NiMet 2021

3.2 Study Population

The study population was based in Kaduna metropolis and survey was carried out across various age groups (0-50 and above) and gender (male and female). The sampling survey was carried out between February-October, 2023, and a total of 1196 Participants (patients) samples were analyzed across different location within the metropolis.

3.3 Sample Size

The sample size (N) was determined using the sample size calculation obtained by employing the prevalence *P. falciparum* HRP2 gene deletion in on study in Equatorial Guinea (Berzosa *et al.*, 2020). The formular described by Daniel *et al.,* (1999) was used:

$$N = \frac{Z^2 P\,(1-P)}{D^2}$$

N (Sample): This represents the target number of total number of participants in the survey

Z (Z Statistic): This represents the statistical level of confidence, typically 1.96 for a 95% confidence level

P (Expected Prevalence): This is the estimated proportion of the total population of participants that exhibit the characteristic of being studied

P = Expected prevalence or proportion (in proportion of one; if 20%, P=0.2)

D (Precision): This is the margin of error or the difference between sample estimate and the true population. The precision in proportion of one: if 5%, d=0.05

Therefore, research sample size:

N = $1.96^2 \times 0.053$ (1- 0.053)/0.0025

N = 77 minimum sample size

Attrition of 10 % of 77 =87

77 samples is the minimum sample size required for this surveillance study.

3.4 Ethical Approval

The ethical clearance and approval for permission to carry out this research was gotten from Kaduna state Ministry of Health.

3.5 Sample Analysis

The blood samples collected from 1196 participants were analyzes using the following techniques:

- Detection of malaria parasite by microscopy
- Detection of PfHRP2 protein by RDT
- Detection of malaria parasite and PfHRP2 gene by PCR
- Estimation of Plasma C-reactive protein (CRP)
- Estimation of Plasma Interferon-gamma (IFN-γ)
- Estimation of Plasma Interleukin-6 (IL-6)

3.6 Specimen Required

- Capillary blood was collected into capillary tube
- Venous blood sample was collected into the anticoagulants(EDTA) and Plain container

3.6.1 Collection by venipuncture

a. Whole blood was collected into collection tube containing anticoagulant (EDTA) by venipuncture.

b. Samples are then stored at 2-8^{O}C when test are not carried out immediately.

c. Stored samples will then be brought to room temperature before testing is carried out.

3.7 Sample Collection and Processing

Sample collection was either by Capillary blood or venous blood samples collection. The capillary blood sample was used for immediate sample processing for making of thick and thin blood film, spotting on whatman 903 papers and on RDT kit.

The venous blood was collected in anticoagulated container for subsequent use

The blood from the patients finger prick was used for malaria diagnosis using both microscopy and RDT.

The blood was spotted on whatman 903 paper (GE Healthcare Bio-Science Corp) for molecular studies.

The blood on the filter paper was air dried and stored in a double zip-lock bag with silica gel at 4C.This was subsequently transferred for PCR diagnostic confirmation. The venous blood collected was also collected when sample may be delayed.

The plasma sample was used for evaluation of CRP and pro-inflammatory cytokine

3.8 Microscopy

3.8.1 Reagent (Stain)

Biolab Giemsa stain

Labtec Giemsa stain

The blood samples for microscopy were made into thick and thin blood films at the site of blood collection. The blood specimen slides prepared were made on a grease-free class slide after collection. It was then allowed to air dry. The films were stained 10 % giemsa stain (Labtec) for 10 minutes. Again each slide was allowed to air, and then examined by an oil immersion objective lens(X100), All fields under the microscope were critically examined before declaring a slide negative or positive.

3.8.2 Thick Film

Each of the thick film microscopic slides were examined for the presence of malaria parasite only.

3.8.3 Thin Film

Each of the thin film slides were examined for speciation only when malaria parasite had been identified on the thick film. Each slide (thick and thin film) was examined independently by two microscopists and the results were recorded as positive when both microscopists found evidence of malaria parasite and identified the same species. In case of any discrepancies between two sides then a third opinion is sort from a third microscopist.

3.9 Rapid Diagnostic Test

The RDT used in situ was Add Malaria Pf Rapid Malaria Detection Test (ADVY CHEMICAL, Thane-400 604 INDIA).This test detects *Plasmodium falciparum* histidine rich-protein2 (PfHRP2) present in the Parasitaemic blood sample.

3.9.1. Assay Description

According to the manufacturer, **"AdvDx Malaria Pf"** test utilizes the principle of Immuno-chromatography. It has the test strip coated with Monoclonal Anti-HRP-II (Test line Pf) which is specific to the histidine rich protein II of *P. falciparum* species. As the test sample flows through the membrane assembly of the device after addition of the buffer solution, the colored colloidal gold, and the anti- HRP-II antibody conjugate complexes with the lysed blood sample. The malarial antigens get immobilized on the respective test lines on the nitrocellulose membrane which leads to the formation of red/purple colored band/s. The unreacted conjugate continues to migrate and is subsequently immobilized at the control "C" region to form a red/purple band. The control band demonstrates that the liquid has migrated, and the assay procedure has been followed correctly. The test has a sensitivity of 96.66% and specificity of 98.5% for

Plasmodium falciparum (Berhane *et al.,*2016).

3.9.2 Principle

The principle is based on immunochromatography, it has the test cassette coated with monoclonal antigen, Anti-HPR2 (Test-line pf) which is specific to the histidine rich protein-2 and it detects HRP2 of *P. falciparum*. As the test sample flows through the membrane assembly of the device after addition of buffer solution, the coloured colloidal gold and the AntiHRP-2 antibody conjugate complexes with lysed blood sample. The malaria antigen gets immobilized on the respective test line on the nitrocellulose membrane which leads to the formation of red band. The unreacted conjugate continues to migrate and is subsequently immobilized at the control 'C' region2-specific proteins for *P. falciparum*. The test has sensitivity of 96.66% for *P. falciparum* and 95.5% and as specificity of 98.42% (Bharti *et al*., 2017).

3.9.3 Analytical Sensitivity

The stated analytical sensitivity (LoD) is 200 Pf/µl of blood for HRP2. To perform the malaria test, 5µl of whole blood is collected with the provided capillary pipette and transferred to the sample well. Four drops of the assay diluent are then added to the diluent well, in accordance with the manufacturer's protocol. The results are read after 15–20 min; only tests containing the control band are considered valid. Participants who's RDTs produced positive results were recommended for treatment.

3.9.4 Performance Evaluation

Based on the demonstrated *P. falciparum* panel detection score (80.0% at 200 parasites/µl), false-positive rates (0.0% for clean negatives, 0.0% for *P. vivax* at 200 parasites/µl, 0.0% for *P. vivax* at 200 parasites/µl) and invalid rate (0.0%), AdvDx Malaria Pf Rapid Malaria Ag Detection Test meets the current laboratory evaluation requirements for this research.

3.9.5 Materials Provided

A. AdvDx Malaria Pf kit

i. AdvDx Malaria Pf test device (cassette) foil pouch with desiccant

ii. Sample dropper

iii. Alcohol swab

iv. Lancet

v. Buffer solution

vi. Product Insert

B. Active ingredients of the main component include:

i. Test cassette embedded with: Gold conjugate: mouse monoclonal antibodies specific to Pf-HRP-2 conjugated to colloidal gold, control line: Goat anti-mouse IgG

ii. Buffer solution: casein, triton x-100 and sodium azide as preservatives

3.10 Specimen Collection For RDT

Test Procedure:

The **AdvDx^{TM} Malaria Pf** kit components and specimens were allowed to attain room temperature.

1. The pouch was opened and device was taken out from it. The device contains a blue control line made up of water soluble dye that disappears as the test runs.
2. The bottle cap of the buffer solution provided with the kit was untightened in the clockwise direction to pierce the dropper bottle nozzle. The buffer bottle was held vertically to ensure that drops contain the correct volume of the buffer.
3. The anti-coagulated blood sample was mixed gently by swirling, and then the sample dropper was dipped into the blood sample to draw 5µL of blood (in the absence of capillary blood).
4. 5µL of blood was loaded into the"SamplePort1(S)"

4 drops (110µL±5µL) of buffer solution was added in to the “Buffer Port 2 (B)”on the test device.

5. The result was read at the end of 20 minutes.
6. The manufacturer literature pamphlet was used to interpret the result.

CAUTION: No result of the test read after 30 minutes, since it may give incorrect results.

Interpretation of the Results:

Whole blood samples may cause red back ground to appear in the result window.

NEGATIVE:

Pf Negative: When only the Purple-colored control band appears indicates no malaria antigens present in the blood sample, meaning no malaria infection, or the number of malaria antigens the blood sample is below the detectable range.

POSITIVE:

Pf Positive: When two bands (“Pf” Test line and Purple-colored “C” Control line) appear within the result window indicates the infection of *P. falciparum.*

The shade of color/intensity of band may vary, but it should be considered positive whenever there is a faint line.

INVALID:

Absence of color band or blue color band at Control line (C) with or without color band at the test line 'Pf' indicates the test is invalid. In this case, please repeat the test using a fresh device and follow the test procedure exactly.

3.11 Molecular Analysis

3.11.1 DNA Extraction

Material required for DNA extraction include;

1. Lysis buffer (NaCl + Detergent)

2. Chromosome condensate

3. Absolute alcohol + 70% alcohol

4. PCR water (Nuclease free wate)

5. Eluent

6. DNA purification kits (e.g., QIAamp DNA Mini Kit, Qiagen)

For PCR detection of PfHRP2 gene:

1. DNA polymerase (e.g., Taq polymerase, Pfu polymerase)

2. PCR buffer

3. $MgCl_2$

4. dNTPs (dATP, dCTP, dGTP, dTTP)

5. PfHRP2 and PfHRP3specific gene primers respectively

6. Probe (e.g., TaqMan probe)

7. PCR master mix

Specific reagents used for PfHRP2 gene detection include:

1. PfHRP2 primer pairs (e.g., HRP2-F and HRP2-R)

2. PfHRP2 probe (e.g., HRP2-P)

3.11.2 Procedure for DNA Extraction

- A 5 mm diameter pouch containing 10 uL of blood was used.
- From the filter paper containing the sample,, the DNA was extracted using commercial (speed tool tissue DNA extraction) kit (Ncogo *et al*., 2015).
- The sample blood cells were lysed using the lysis buffer then
- Chromosome condensate was obtained after lysing the cell

- The DNA was precipitated using absolute ethanol
- It was transferred to filter column
- It was then washed with 70% ethanol
- The PCR water was added to the column

3.12 Molecular Analysis for the Detection of Malaria Parasite

The nested multiplex polymerase chain reaction (NM-PCR) was used to target selected gene encoding 18S subunit ribosomal RNA (ssr RNA). This include an internal amplification control to avoid false negative (18S human rRNA) (Rubio *et al.,* 2002, Ta *et al.,* 2011 and Ta *et al.,* 2014) This molecular analyses was carried out on all the 694 participants blood samples that tested negative by RDT collected for this survey. Of the 694 RDT negative samples, 611 samples shows no amplification in the NM-PCR while 83 samples shows DNA amplification. When there is DNA amplification, it means the Pfhp2 gene is present while in the absence of Pfhp2 gene (deletion) amplification means the gene is absent. Therefore, in the absence of PfHRP2 gene amplification, this means that there is gene deletion. After the NM-PCR analytical tests had been completed on all the 83 RDT- negative samples (83 microscopic positive and 611 negative) was identified as having PfHRP2 gene deletion, positive for malaria by microscopy and PCR. These 83 RDT-negative samples are therefore, classified as RDT false-negatives samples.

3.13 Molecular Analysis for Pfdhfr, Pfdhps, Pfmdr1 and Pfcrt Genes

The nested polymerase chain reaction (N-PCR) test was carried out to study these genes; *Plasmodium falciparum* dihydrofolate reductase gene (Pfdhfr), *Plasmodium falciparum* dihydroptereoate *(*Pfdhps), *Plasmodium falciparum* multi- drug resistant 1 gene (Pfmdr1) *and Plasmodium falciparum chloroquine resistance transporter gene (*Pfcrt) in the 83 RDT false-negative samples. This N- PCR serve as control so, when these genes are detected it means the PCR analysis was successful. The following gene fragments which

include *Pfdhfr* (108/164, 51/59), *Pfdhps* (400 and 500), *Pfmdr1* (86/1246) and *Pfcrt* (76) were studied using Maryland protocol. This analytical procedure was used as control for qualitative detection of the DNA in the 83 RDT false-negative samples (Benito *et al*, 1994). Hence if all the samples were diagnosed by nested-PCR for these PfHRP2 and PfHRP3 genes, this therefore indicates that the DNA can be qualitatively detected. Therefore, if exon2 amplification fragment is obtained from the PfHRP2 and PfHRP3 genes, then it was not due to poor DNA quality or other factors, but because there was true deletions in PfHRP2 and PfHRP3 genes.

3.14 Molecular Analysis for exon2 of PfHRP2 and PfHRP3 genes.

Of the 83 RDT false-negative samples with its entire malaria parasite-DNA confirmed using NM-PCR and the *Pfdhfr, Pfdhps, Pfmdr1 and Pfcrt,* N-PCR were again used for the amplification of the exon2 of PfHRP2 and PfHRP3 genes. This will allow for the detection of presence or absence of these set of two genes (Baker, 2005, Ugah et al 2017 and Kozycki et al 2017).The analyses was performed using the same primer but with minor adjustment. The primer sequences for the N-PCR were as follows:

PfHRP2-F1 (5′-CAAAAGGACTTAATTTAAATAAGAG-3′) and

PfHRP2-R1 (5′-AATAAATTTAATGGCGTAGGCA-3′) was designed for primary reaction to anneal to the 5′ and 3′ ends of exon 2 of *PfHRP2*. Semi-nested amplification was performed for secondary reaction by use of the primers

PfHRP2-F2: (5′-ATTATTACACGAAACTCAAGCAC-3′) and PfHRP2-**R2** serves as the reverse sequence of the PCR.

The same procedures and conditions were used to amplify the PfHRP3 gene by use of the primers

Outer primer: PfHRP3-F1 (5′-AATGCAAAAGGACTTAATTC-3′),

PfHRP3R1 (5′ TGGTGTAAGTGATGCGTAGT-3′) and

Inner primer: PfHRP3-F2 (5′-AAATAAGAGATTATTACACGAAAG-3′) and R2 is the reverse primer.

The PCR cycling conditions

Thermocycling conditions for amplification of PfHRP2 and PfHRP3 Genes

Species		**Initial Denaturation**	**Denaturation**	**Annealing**	**Extension**	**Cycles**	**Final extension**	**Hold**
PfHRP2	Outer	95°C for 5 minutes	95°C for 30 seconds	55°C for 1 minute	72°C for 1 minute	30	72°C for 3 minutes	4°C
	Nested	95°C for 5 minutes	95°C for 30 seconds	55°C for 1 minute	72°C for 1 minute	30	72°C for 3 minutes	4°C
PfHRP3	Outer	95°C for 5 minutes	95°C for 30 seconds	55°C for 1 minute	72°C for 1 minute	30	72°C for 3 minutes	4°C
	Nested	95°C for 5 minutes	95°C for 30 seconds	55°C for 1 minute	72°C for 1 minute	30	72°C for 3 minutes	4°C

The PCR cycling conditions were as follows:

Heat denaturation: This is the first stage of the analytical process, the 1st PCR and 2nd PCR at 95°C for 15 min, followed 30 cycles by 95°C for 1 minute.

Annealing of primers: at 55°C for1 minute and 72°C for 1 minute and final extension at 72°C 10 min. This was done for both genes.

Positive control: The 3D7 *Plasmodium falciparum* strain parasite was used as a positive control for *PfHRP2*

Negative control*: The* Dd2 *Plasmodium falciparum* strain was used as a negative.

The 3D7 strain is known to have all *PfHRP2*and *PfHRP3* genes, and the relevant flanking genes, while Dd2 lacks both *PfHRP2/3* and its flanking genes. All the positive amplifications of *PfHRP2* and *PfHRP3*genes (exon2) were sequenced from opposite directions using forward and reverse primers of exon2. PCR products were purified using GeneJet PCR cleanup kit

Confirmation of Gene Deletion

The presence of parasites in the RDT false-negative samples was verified by microscopic positive samples with positive results for 18SrRNA but negative results were considered as PfHRP2-deleted after excluding low parasitaemia to exclude incorrect deletion calls**.**

Agarose Gel Electrophoresis

The Agarose gel (2% w/v) was used to confirm the presence of bands. The PCR amplified products (amplicons) were detected by running 10 µL of the PCR mixture on the agarose gel, stained with 0.5 µg/mL ethidium bromide solution. The samples were run using a Powerpack (Biorad, CA, USA) at 99 volts for 30 min alongside a 100 bp DNA ladder and a negative control (autoclaved distilled water), followed by separation. The bands were visualized under a UV transilluminator (Bio-Rad).

3.15 Molecular Assay of Interleukins

3.15.1 Enzyme-Linked Immunosorbent Assay

Human Interleukin 6 (IL-6) ELISA KIT: Is a ready-to-use microwell, strip plate ELISA (Enzyme-linked immunosorbent assay) kit used to detect and quantify interleukin 6 (IL-6)

Specification

Assay Type: Solid Phase Sandwich ELISA

Specificity: Quantitative determination of Human IL-6

Assay Range: 3.13 - 200 pg/mL

3.15.2 Materials Provided

1. Capture Antibody: 1 mg/mL of mouse anti-Human IL-6 monoclonal antibody. 2 µg/mL is diluted in PBS before coating.

2. Detection Antibody: 0.2 mg/mL of mouse anti-Human IL-6 monoclonal antibody conjugated to horseradish-peroxidase (HRP).

The 0.25 µg/mL is diluted in dilution Buffer before use.

3. Standard: 35 ng of recombinant Human IL-6.This is Reconstituted with 1 mL Dilution Buffer and stored at -20℃ to -80℃.

3.15.3 Principle of IL-6 ELISA Assay

This assay is based on a solid-phase sandwich enzyme-linked immunosorbent assay. The following protocol are observed

- Specific Binding: The assay uses two antibodies that specifically bind to human IL-6.
- One antibody is immobilized on a plate (capture antibody), while the other is conjugated to an enzyme for antibody detection.
- IL-6 Capture: When a sample containing IL-6 is added, the capture antibody binds to IL-6
- Detection: The detection antibody, conjugated to an enzyme (e.g., HRP), binds to a different epitope on IL-6, forming a sandwich complex.

- Signal Generation*: The enzyme converts a substrate into a detectable signal (e.g., colour change).
- Quantification: The signal intensity is directly proportional to the concentration of IL-6 in the sample. By comparing the signal to a standard curve, the concentration of IL-6 can be determined.

3.15.4 Assay Procedure

1. 100 µl of each standard and sample were added into the appropriate well and covered properly.

2. This was then incubated for 2.5 hours at room temperature.

3. The solution was discarded and washed 4 times with 1x was solution (300 µl) using a multi- channel pipette or autowash.

4. The pipette was inverted and blurted against a clean white filter paper.

5. 100 µl of 1x biotinylated 1L-6 antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking

6. The solution was discarded and step 2 wash was repeated

7. Then 100 µl of 1x HRP- Streptavidin solution was added to each well and incubated for 45 minutes at room temperature with gentle shaking.

8. The solution was discarded and step 2 wash was repeated.

9. 100 µl of TMB substrate reagent was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking.

10. 50 µl of stop solution was added to each well, and was finally read immediately at 450nm.

Precaution

-All materials and reagents were prepared and equilibrated at room temperature (18-25^{0}C) before use.

-Assay of all standard, control and sample were done in duplicate.

CHAPTER FOUR
RESULTS

A total of 1196 blood samples of participants were first diagnosed by RDT, followed by microscopy and then NM-PCR. The *Plasmodium* samples marked as negative by both microscopy and RDT were tested by NM-PCR as a quality control of the diagnoses. Of this group of RDT negative samples, (n-694), 83 (6.9%) were identified as fable-negatives by PCR and were diagnosed as 78 *P. falciparum*, 2 *P. malaria*, 2 *P.ovale* and 1 mixture of *P. falciparum/P.vivax.*

Figure 4.1 shows the flow chart of the systematic steps of how the 1196 participants samples collected were processed and analysed.

The 83 RDT negative samples could have been due to; deletion in Pfhrp 2/3 gene deletion, low parasitaemic level below detection limit the RDT, non-*falciparum* species and technician error in interpretation of result.

Figure 1

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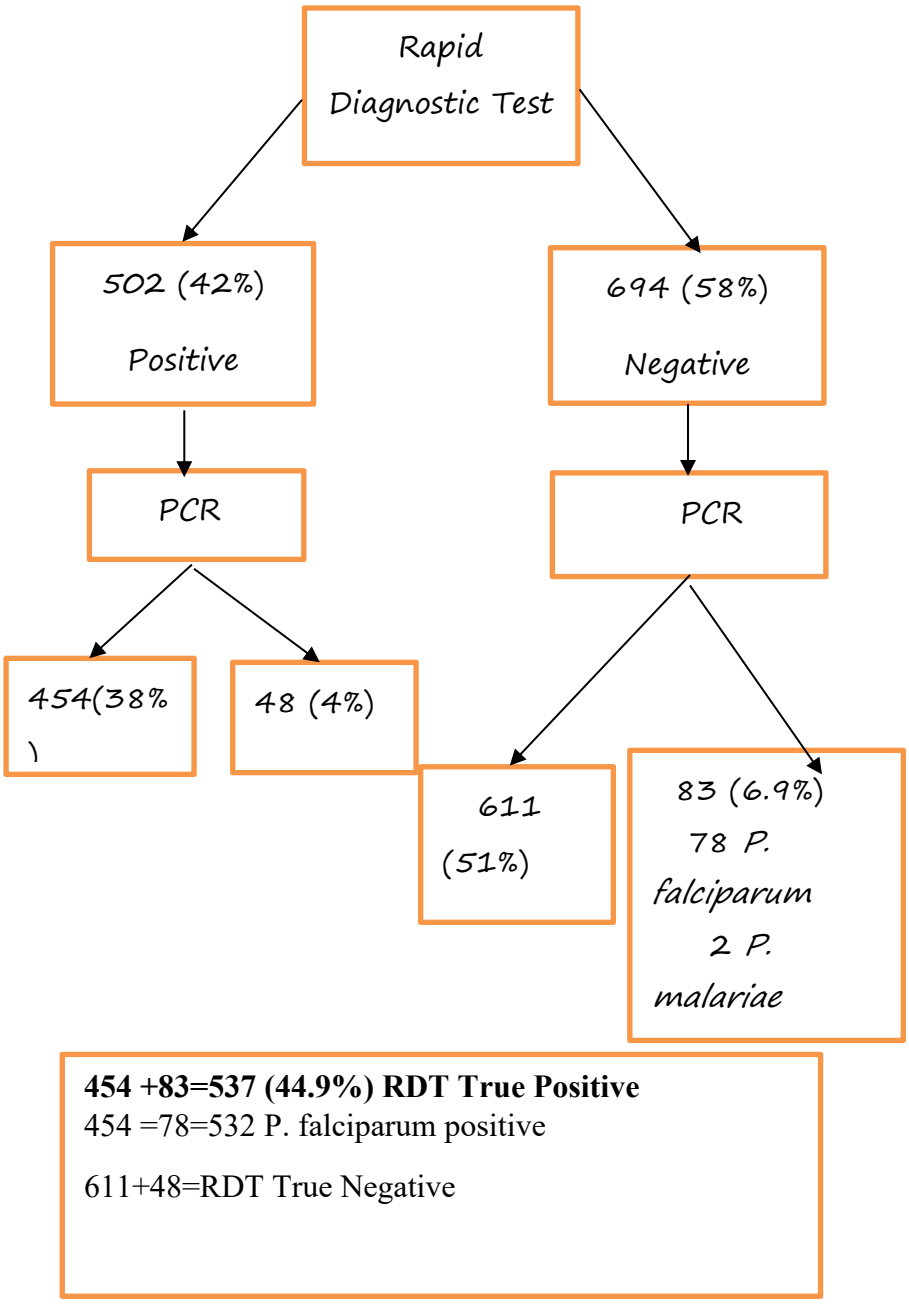


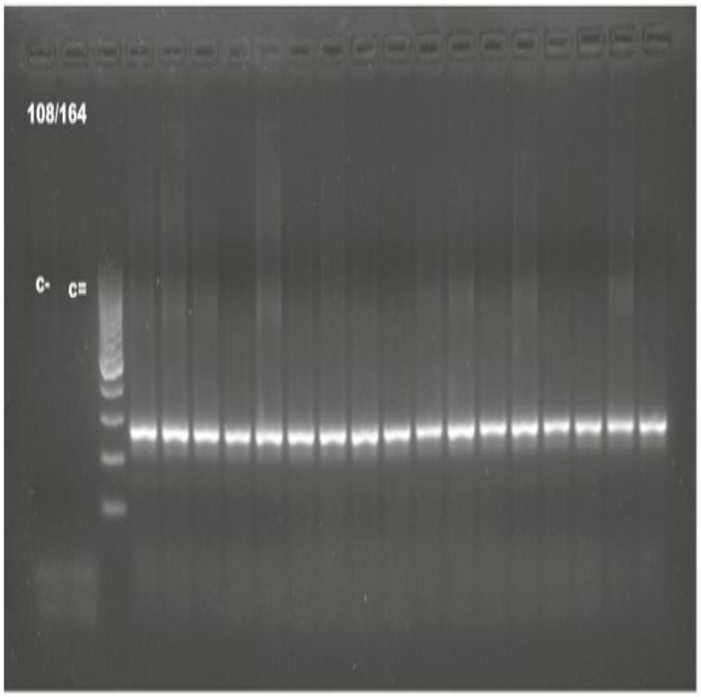
Figure 4.1: Flowchart showing analysis of 1196 samples: 502 of the total samples were Negatives by RDT and 694 positives. Of the negatives, PCR detected 83 (78 Pf, 2 Pm, 2 Po and 1 Pf/Po) as positives, hence classified as RDT false-negative. Frequency of false negative by RDT is 6.9%.

The DNA from the 83 RDT false-negative was correctly amplified by N-PCR for the following *P. falciparum* genes amplifications (Figure 4.2) Pfdhfr (108/164) and 51/59 , product size 254 bp and 113 bp respectively, Pfdhps (400 and 500, product size 148 and 201 bp respectively), Pfmdr1 (86 and 1246, product size 203 bp and 295 bp respectively) and Pfcrt (76 product size 145 bp). This PCR analytical results indicate that the DNA was extracted was intact with no inhibition factors present and has adequate concentration needed for successful PCR for exon^2 of PfHRP2 and PfHRP3 genes.

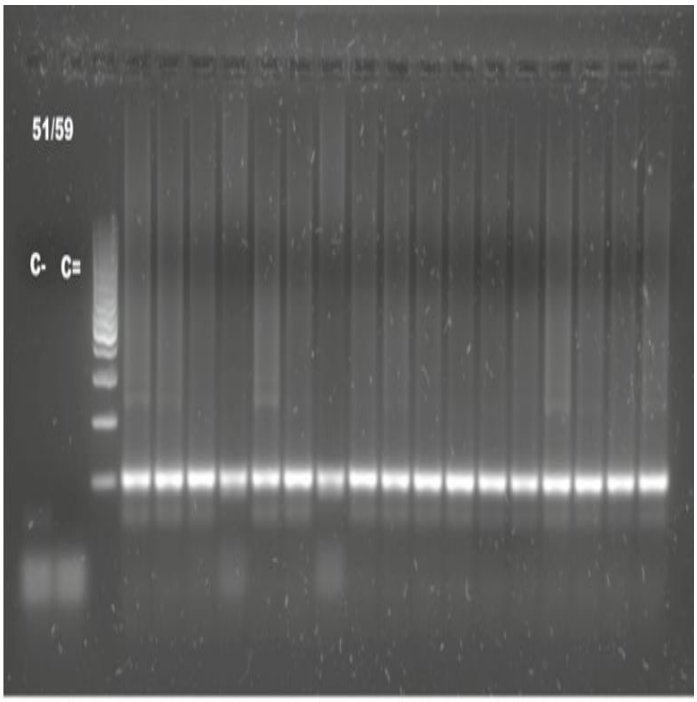
All the DNA samples were analysed for deletion of PfHRP2 and PfHRP3 genes using PCR (for exon2 of PfHRP2 and PfHRP3); the size of the expected fragment if amplification did occur were ± 814 bp for PfHRP2 and ±719 bp for PfHRP3; this determined the presence or absence of these genes in the samples.

Figure 4.2 shows the result of Nested-PCR test. The PCR analysis for exon2 for PfHRP2/3 was done on all samples that are negative by RDT and positive by NM-PCR, although some samples were diagnosed as non-falciparum. In the five (5) non-*falciparum* samples, no amplification fragment was obtained. This indicate that the specificity of PCR for exon2 for PfHRP2/3 since it only amplify these gene of P.falciparum.

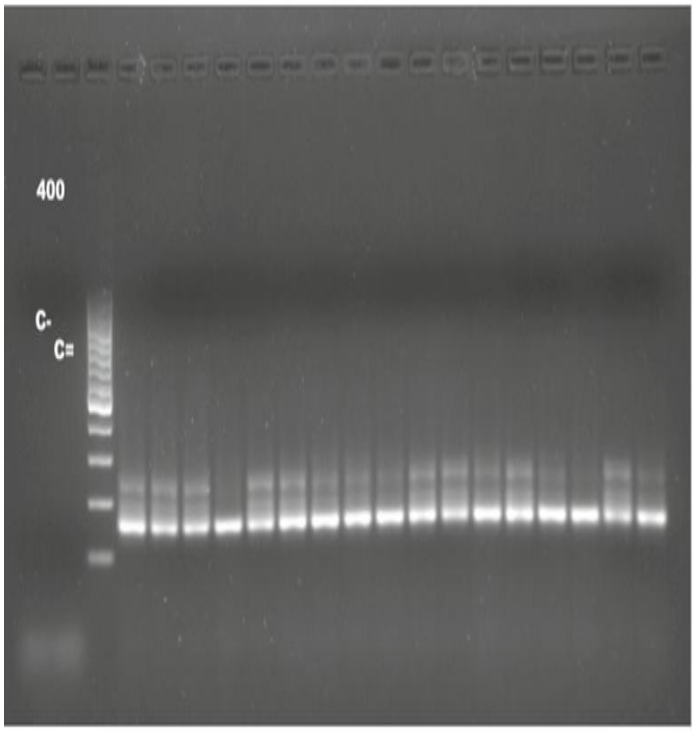
PCR-*pfdhfr-108/164*



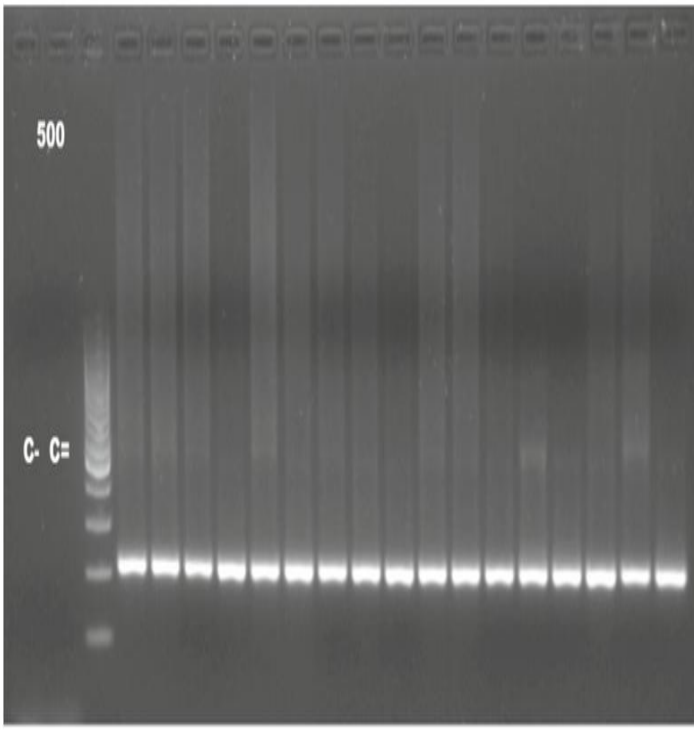
PCR-*pfdhfr-51/59*



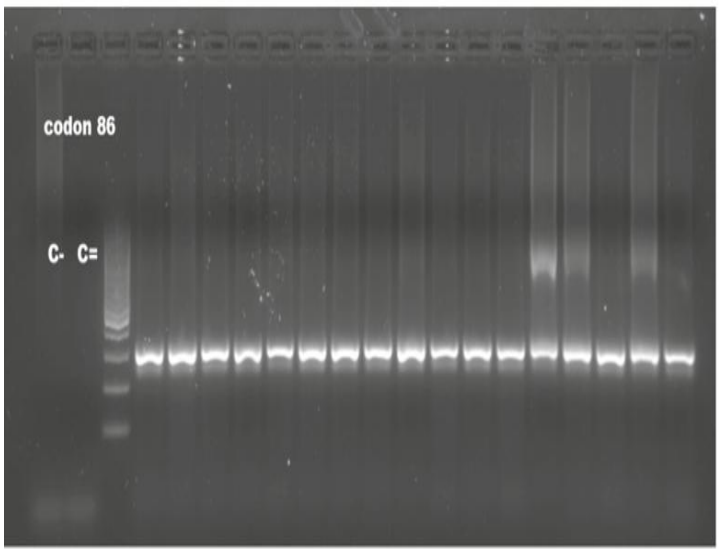
PCR-*pfdhps-*



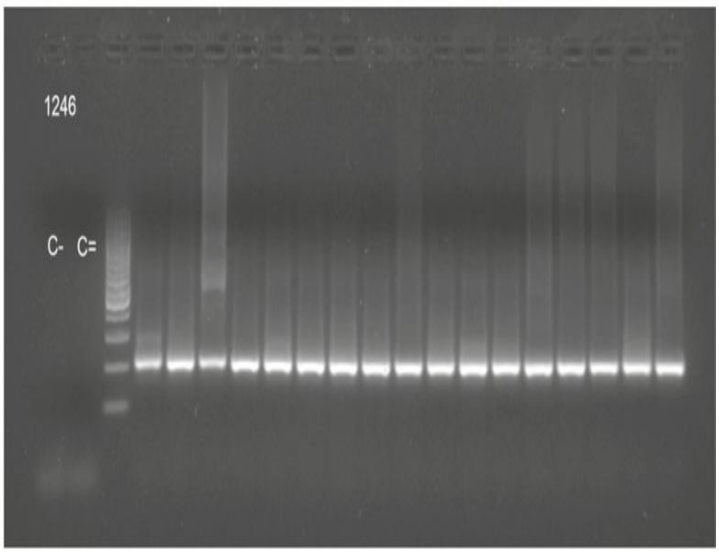
PCR-*pfdhps-500*



PCR-pfmdr1-86



PCR-pfmdr1-1246



PCR-pfcrt-76

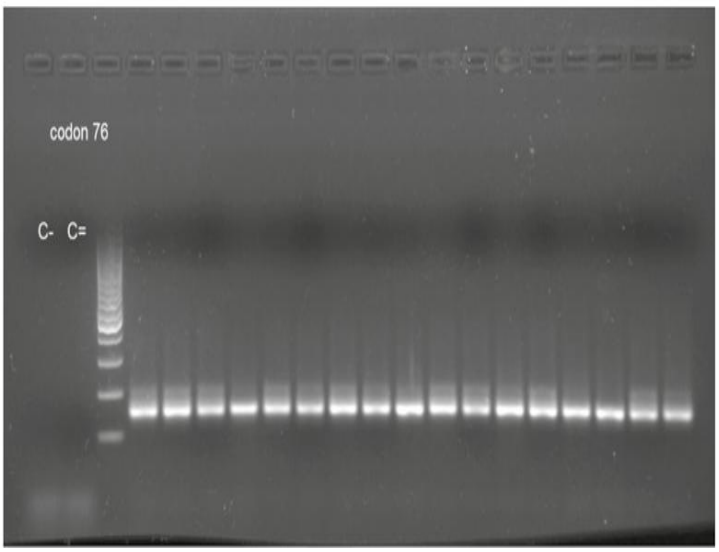


Figure 4.2 Results of the Nested PCR for Pfdhfr, Pfdhps, Pfmdr1 and Pfcrt genes: amplification appears in all cases, therefore, it indicates that the DNA was well extracted and works correctly in PCR. These PCRs are used as a control, all samples amplified perfectly so when no amplification appears in PfHRP2/3, this indicates that there is deletion, it is not a problem with the DNA.

After performing the PCR on 83 RDT false-negative samples, 5 non-*falciparum* species;2 P. malariae, 2 *P. ovale* and 1 mixture *P. falciparum/P .ovale* was negative in the PCR for these two genes, therefore, was detected deletion in these genes for *P.falciparum*.

In the remaining 78 samples which were *P. falciparum;* 69 samples (5.8%, 95% CI 5.26-7.74) were identified out of 1196 to have deletion in both genes (Table 4.10). Therefore, the amplification fragment was absent. Five samples (0.42% 95% CI 0.-48-1.64) had no identified deletion in any of the genes studied. 4 samples (0.33% 95% CI -0.96-1.54) have deletion only in PfHRP2 only but not in PfHRP3, 5 (0.42%, 95% CI -0.48-1.64samples have deletions only in PfHRP3 but not in PfHRP2

The five samples were also identified in the film microscopy to be non-*falciparum* species. Microscopy identified *P. malariae, P. vivax* and *P. ovale*. In this case, no amplification fragment were identified.

However, considering only the PfHRP2 gene, (RDT detects the PfHRP2 protein) within the total 1196 samples, 73 samples (6.1% 95% CI 4.76-7.44) had deletion. In the mixed infection, *P. falciparum* and *P. malariae,* based on NM-PCR, neither the PfHRP2 nor PfHRP3 gene were detected.

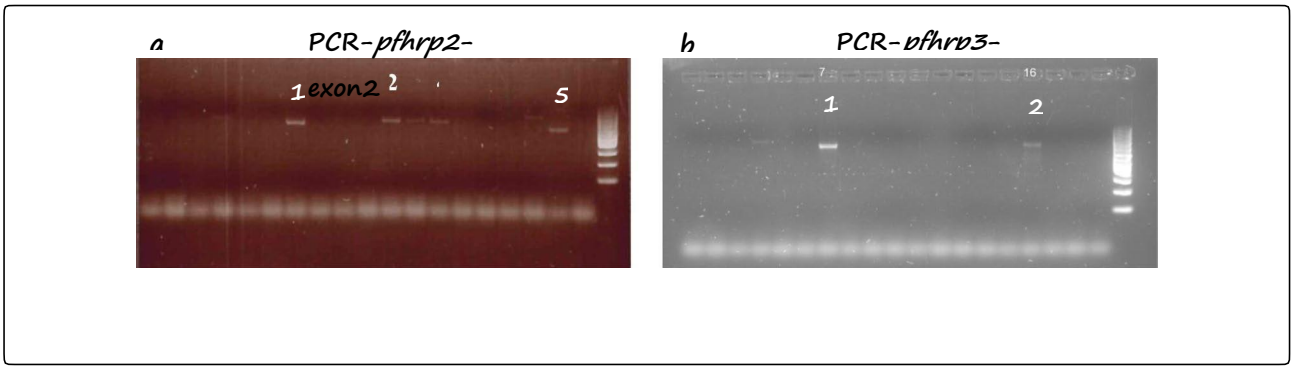


Figure 4.3: Result of the Nested PCR for *PfHRP* 2/3; the presence of the amplification fragments indicates the presence of the gene. Fig a: lines 1/2/3/4/5 (± 814 bp *PfHRP* 2) and fig b: lines 1/2 (± 719 bp *PfHRP* 3). When the fragments do not appear indicates that deletion exists: fragments are sequenced to confirm that they correspond to the *PfHRP* 2/3 genes.

Table 4.1 Shows table of Microscopy and NM-PCR Result for Detection of *P. falciparum.* From 1196 participants 694 samples were negative by RDT. Upon further diagnosis by microscopy, 83 samples were found to be positive in all the RDT negative samples when examined by thick film and this finding was confirmed by PCR. Also by examination of thin film through the microscope, of the 83 positive samples, 78 were found to be *P. falciparum* species and five was non-*falciparum* species. From this finding, of the 694 RDT negative, 611 were negative by microscopy and PCR and 83 were RDT false negative.

Figure 4.4 shows bar chart distributions results obtained by RDT, microscopy and NM-PCR. In the bar chart diagrams, the examination bars represent the 694 RDT- negatives (maroon bar) examined further by microscopy (blue bar) and NM-PCR (green bar). The positive bars represent positive result obtained from examination of 694 RDT-negatives, 83 samples were found to be positive by microscopy (blue) and PCR (green). Therefore 611 were RDT-negatives and 83 samples were RDT false-negatives. Also from the 694 RDT-negatives, 611 samples were negatives by microscopy and NM-PCR. The negative bars represent 611 by microscopy, blue bar and NM-PCR, green bar. This bar chart represent all the three diagnostic method was carried out on all the 694 negatives (blue, maroon and green), 83, 611 and 83 were positive respectively while 611 was negative for blue and green barcharts respectively.

Table 4.1: Microscopy and NM-PCR Result for Detection of *P. falciparum*

Variables	Examined	Positive	Negative	χ^2	*P*
Microscopy	694	83	611	324.17	<0.001
NM-PCR	694	83	611		

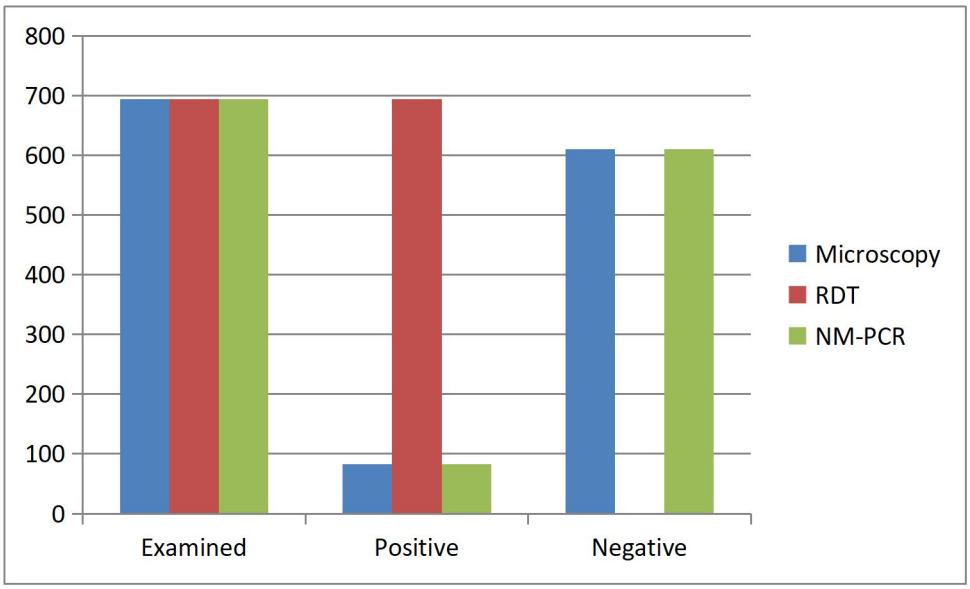


Figure 4.4: Bar Chart Distribution of Microscopy, RDT and NM-PCR Negative Results

Key.

Blue colour = Microscopy

Maroon colour = RDT

Green colour = NM-PCR

Figure 4.5 shows pie chart distribution of RDT positive and negative results. Of the 1196 participants samples diagnosed by RDT, 694 represent 58% of the negative samples (maroon colour) while and 502 represent 42% of the positive samples (blue colour) on the pie chart.

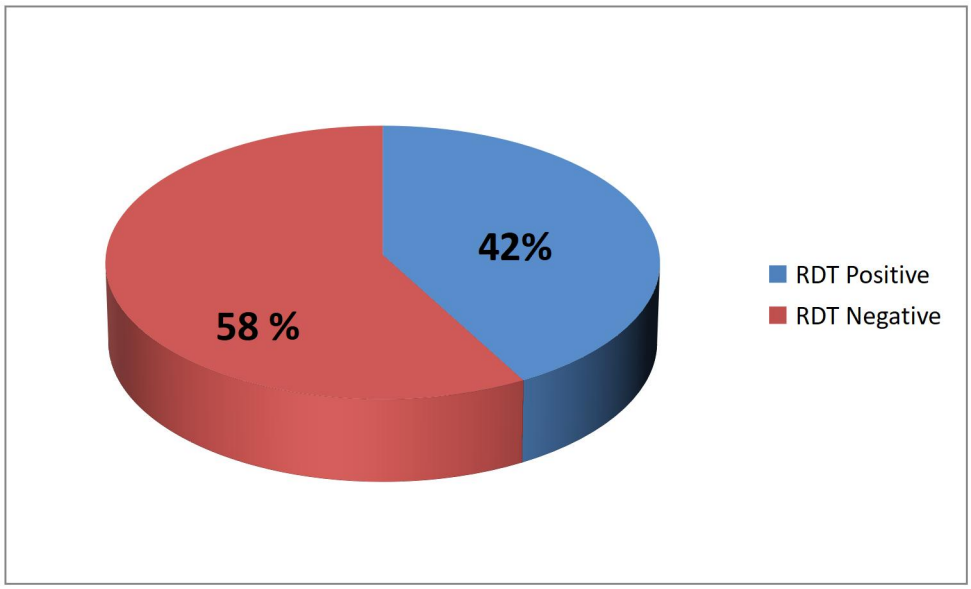


Figure 4.5: Pie Chart RDT Positive and Negative Results in Percentage of Total Number of Participants

Keys:

Blue Colour = RDT Positive

Maroon Colour = RDT Negative

Table 4.2 shows RDT Negative results based on age group and gender of the patients. Across all age groups, the 0–10 years category recorded the highest proportion of RDT-negative results (29.5%), followed by the 41–50 years group (16.6%) and the >51 years category (11.8%). The 11–20, 21–30, and 31–40 age groups each contributed roughly similar proportions, ranging from 13.3% to 14.6%. A chi-square test revealed a statistically significant association between age-group distribution and RDT-negative results ($\chi^2 = 87.90$, $p < 0.001$), indicating that the likelihood of testing negative varied significantly across age categories. Based on gender distribution, of the 694 negative samples; the number of males who were positive are significantly higher ($\chi^2 = 3.90$, $P = 0.048$) 373 (53.8%) compared to females and 321 (46.3 %).

Age Group	n	MALE	FEMALE	TOTAL	χ^2	*P*
0-10	205	110 (29.5%)	95 (29.6%)	205 (29.5%)	87.90	<0.001
11-20	101	50 (13.4%)	51 (15.9%)	101 (14.6%)		
21-30	99	54 (14.5%)	45 (14.0%)	99 (14.3%)		
31-40	92	49 (13.1%)	43 (13.4%)	92 (13.3%)		
41-50	115	57 (15.3%)	58 (18.1%)	115 (16.6%)		
≥51	82	53 (14.2%)	29 (9.0%)	82 (11.8%)		
	TOTAL	373(%)	321(%)	694(%)		

Table 4.2: RDT Negative Result Based on Demographic Distribution

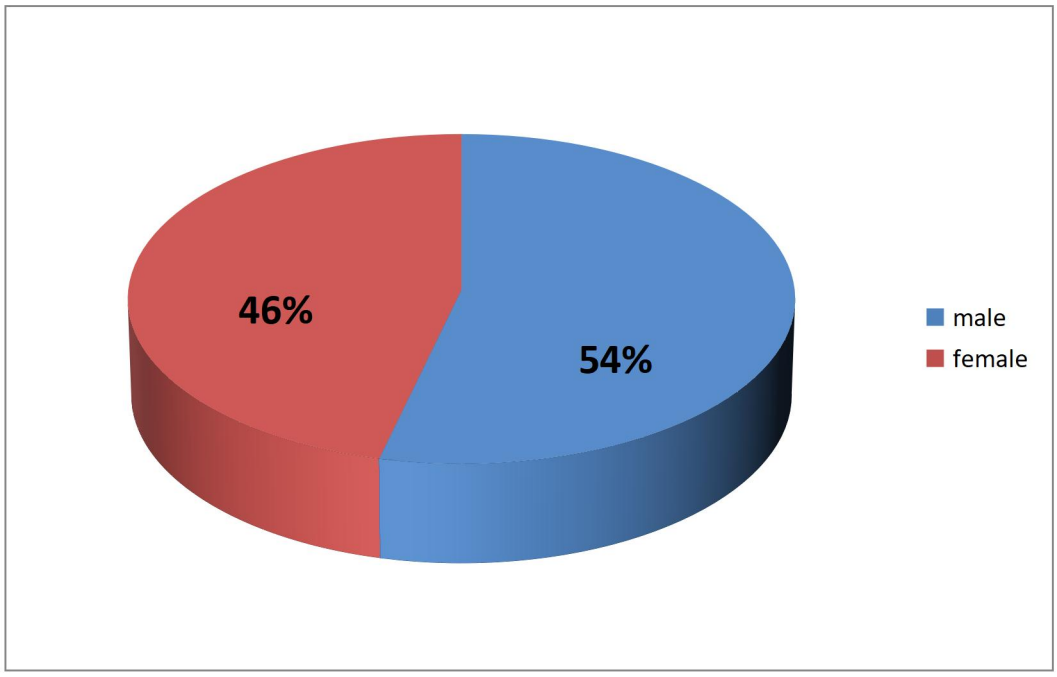


Figure 4.6: Pie Chart Showing Gender Distribution of RDT Negative Result

Keys.

Blue colour = Male

Maroon colour = Female

Table 4.4 shows the distribution demographic of parameters for RDT positive and negative results obtained from all the participants. Out1196 samples for this study, 670 (56%) were males and 526(44%) were females. Out of these, 670 samples diagnosed were for male, 95 (14.2%) were in age group 0-1, 114(17%), 99(14.8%), 105(15.7), 88(13.1), 98(14.6%) and 71(10.6%) for the age bracket 2-4, 5-12, 13-19, 20-39, 40-59, and >60 respectively. Out of the 526 female samples, 76(14.5%) were within the age group 0-1, 81(15.4%), 80(15.2%), 68(12.9%), 96(18.3%), 80(15.2%) and 45(8.6%) for the age group 2-4, 5-12, 13-19, 20-39, 40-59, and .>60 respectively. These (male and female) gives a total of 1196 samples diagnosed by RDT.

Out of the 670 male samples, the RDT male positive samples were; 95(43%) participants in age group 0-1, 114(51%), in 2-4, 99(49.5%) 5-12, 105(48.5%) (15.7) 13-19, 88(50%) in 20-39, 98(41.8%) in 40-59 and 53(25.%) in >60. For the 526 female participants, RDT positive samples were 76(55%), 81(43%), 80 (56.9%), 68(51.1%) 96(55.2%) 80(27.5) and 45(35.6) for the age bracket 2-4, 5-12, 13-19, 20-39, 40-59, and >60 respectively.

Table 4.3 presents the demographic distribution of RDT-positive and RDT-negative results across different age groups for both males and females. Among males (n = 670), the highest proportion of test results occurred in the 0–10-year age group, which accounted for 31.2% of all male cases, with 99 testing positive and 110 testing negatives. This was followed by the 21–30-year group (15.7%), the 41–50-year group (14.6%), and the 11–20 and 31–40-year groups, each contributing approximately 13–15% of male cases. The ≥51-year group represented the lowest male proportion at 10.6%. Among females (n = 526), a similar pattern was observed, with the 0–10-year age group contributing the highest proportion (23.0%), followed by the 31–40-year group (18.3%), the 41–50 year group (15.2%), and the 11–20 and 21–30-year groups at 15.2% and 12.9%, respectively. The ≥51 age group accounted for the smallest proportion among females (8.6%). The chi-square tests revealed statistically

significant associations between age distribution and both RDT-positive and RDT-negative results for males ($\chi^2 = 73.28$ and 44.83, respectively; $p < 0.001$ for both) and for females ($\chi^2 = 47.51$ and 47.32, respectively; $p < 0.001$ for both), indicating that the likelihood of testing positive or negative differed significantly across age groups for both sexes.

Table 4.3: Demographic Distribution of RDT Positive and Negative Results

Age Group	RDT (+ve) Male	RDT (–ve) Male	Male Total (%)	RDT (+ve) Female	RDT (-ve) Female	Female Total (%)
0-10	99	110	209 (31.2)	59	95	154 (23.0)
11-20	49	50	99 (14.8)	29	51	80 (15.2)
21-30	51	54	105 (15.7)	23	45	68 (12.9)
31-40	39	49	88 (13.1)	53	43	96 (18.3)
41-50	41	57	98 (14.6)	22	58	80 (15.2)
≥51	18	53	71 (10.6)	16	29	45 (8.6)
Total	297	373	670	205	321	526
χ^2	73.28	44.83		47.51	47.32	
P	<0.001	<0.001		<0.001	<0.001	

Figure 4.5 show the distribution of RDT positive result based on Age and Gender. Of the 502 RDT positive result, the age group 0-10 had the highest (161) of P. falciparum infection while those above the age of 50 had the lowest (34).

Table 4.4 presents the distribution of RDT-positive results across different age groups for both males and females. A total of 502 individuals tested positive, comprising 297 males and 205 females. The highest proportion of positive cases occurred in the 0–10-year age group, which accounted for 161 cases overall, representing the largest share among both males (33.3%) and females (30.2%). This was followed by the 31–40-year group with 92 positive cases, driven largely by females (25.9%). The 11–20 and 21–30-year groups contributed 78 and 74 positive cases respectively, while the 41–50-year group accounted for 63 cases. The lowest proportion of RDT-positive results was observed in individuals aged ≥51 years, contributing only 34 cases overall. A chi-square value of 108.40 with a p-value < 0.001 indicates a statistically significant association between age group and RDT-positive results, suggesting that age significantly influenced the likelihood of testing positive.

Table 4.4: Distribution of RDT +VE Result Base on Age

Age Group	MALE	FEMALE	TOTAL +VE	X^2	p-value
0-10	99 (33.3%)	62 (30.2%)	161	108.40	<0.001
11-20	49 (16.5%)	29 (14.2%)	78		
21-30	51 (17.5%)	23 (11.2%)	74		
31-40	39 (13.1%)	53 (25.9%)	92		
41-50	41 (13.8%)	22 (10.7%)	63		
≥51	18 (6.0%)	16 (7.8%)	34		
TOTAL	297	205	502		

Key: Statistical Analysis and Inference

Table 4.5 show the percentage of RDT result based on age distribution. Of 1196 participants blood samples diagnosed by RDT, the age group 0-10 had the highest number (366, 30.6%) of participants infected by malaria while the age group of 50 and above had the lowest (116, 9.7%). This table displays percentage age distribution of positive and negative RDT result with no hypothetical testing.

Table 4.5: Percentage of RDT Result based on Age Distribution

Age Group	RDT (+) TOTAL	RDT (-) TOTAL	TOTAL (-VE + +VE)
0-10	161 (32.1%)	205 (29.5%)	366 (30.6%)
11-20	78 (15.5%)	101 (14.6%)	179 (15.0%)
21-30	74 (14.7%)	99 (14.3%)	173 (14.5%)
31-40	92 (18.3%)	92 (13.3%)	184 (15.4%)
41-50	63 (12.6%)	115 (16.6%)	178 (14.9%)
≥51	34 (6.8%)	82 (11.8%)	116 (9.7%)
TOTAL	502	694	1196

Key

RDT= Rapid Diagnostic Test.

Table 4.6 presents the diagnostic comparison between the Rapid Diagnostic Test (RDT) and the confirmatory PCR test, which is considered the gold standard. Out of the 537 PCR-confirmed positive cases, the RDT correctly identified 454 as positive (true positives) but missed 83 cases (false negatives). Similarly, among the 659 PCR-confirmed negative individuals, the RDT accurately classified 611 as negative (true negatives), while 48 were incorrectly classified as positive (false positives). Overall, the table shows that the RDT demonstrated reasonably good agreement with PCR, although some misclassification occurred, particularly in the form of false negatives.

Table 4.7 summarizes the diagnostic performance indicators derived from the RDT–PCR comparison. The RDT shows a sensitivity of 85%, meaning it correctly detects 85% of true disease cases. Its specificity of 93% indicates high accuracy in identifying individuals without the disease. The overall accuracy is 89%, suggesting that the test performs well across both positive and negative classifications. The positive predictive value (90%) implies that individuals who test positive have a high likelihood of truly having the disease, while the negative predictive value (88%) indicates that most individuals who test negative are indeed disease-free. The post-test probabilities mirror these results: a positive test yields a 90% probability of actual disease, whereas a negative test reflects an 88% probability of being healthy. These results collectively demonstrate that the RDT is a reliable screening tool when compared to PCR, with particularly strong performance in ruling out disease.

Table 4.6: Diagnostic Agreement Between the Rapid Diagnostic Test (RDT) and Gold Standard PCR Test.

	Gold (PCR) Positive	Gold (PCR) Negative	Total
Test Positive	454	48	502
Test Negative	83	611	694
Total	537	659	1196

Table 4.7: Diagnostic Performance Metrics of the Rapid Diagnostic Test (RDT) Compared With PCR

Sensitivity	**85 %**
Specificity	93 %
Accuracy	89 %
Positive Predictive Value	90 %
Negative Predictive Value	88 %
Post-test Disease Probability	90 %
Post-test Health Probability	88 %

Figure 4.7: Pie Chart Showing Percentage Distribution of RDT Negative and False-Negative RDT Results

Key

Blue Colour = RDT Negative

Green Colour = RDT False-Negative

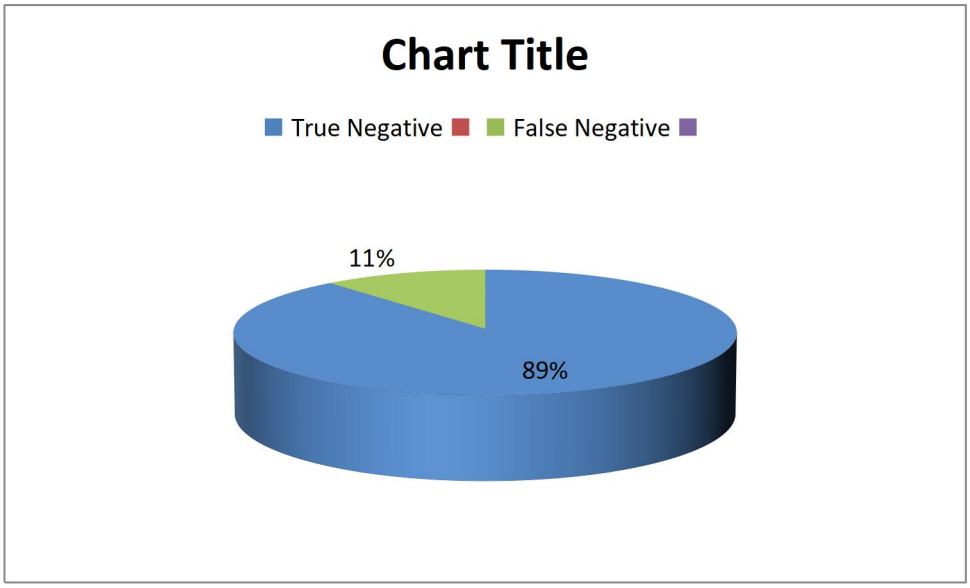


Figure 4.7 Pie chart showing True RDT positive and True RDT Negative Result Distribution in Percentage

Table 4.8 shows demographic distribution of prevalence of PfHRP2 gene deletion. Of the 73 sample that have PfHRP2 gene deletion, the table shows the highest prevalence amongst age 0-10 accounting for 28%. This is followed by the age group 11-20 (20.8%). The age group with the least prevalence of gene deletion is 31-40 (10.4%) followed by 41-50 (11.7%).

This finding also shows that prevalence of gene deletion is higher in male (53.4%) compared to female (46.6%) and highest in children under 10 years compared to other age groups. Chi square test of association indicates statistical insignificant association ($\chi^2 = 4.60$, $P = 0.331$) between PfHRP2 deletion and age.

Table 4.8: Demographic Distribution of Prevalence of PfHRP2 deletion

	Number of deletions				
	Males	Females	Total		
Age Group	n (%)	n (%)	n (%)	χ^2	*P*
0-10	11 (55)	9 (45)	20 (27.4)	4.60	0.331
11-20	9 (56.3)	7 (43.7)	16 (21.9)		
21-30	4 (40)	6 (60)	10 (13.6)		
31-40	4 (50)	4 (50)	8 (11.0)		
41-50	5 (62.5)	3 (37.5)	8 (11.0)		
≥51	6 (54.5)	5 (45.5)	11 (15.0)		

Table 4.9: Shows samples in which deletion was detected inPfHRP2 and PfHRP3 genes. From the 83 RDT negative samples; 69 samples were discovered to have deletions in both genes, 4 had deletion only in PfHRP2 and 5 had deletion only in PfHRP3. However, 73 samples have deletion in PfHRP2 and 74 samples have deletion in only PfHRP3. The total deletion due to Phrp2 and PfHRP3 is 78 samples. In the case of mixed infection 5 had no deletion in both genes.

Table 4.9: Samples in which deletion was detected in *PfHRP2* and *PfHRP3* gene

Samples	No of Samples	PfHRP2	PfHRP3	N=1196 (%)	95% CI	N=537 (%)	95% CI
P. falciparum (N=83)	69	D	D`	5.80	4.6-7.0	12.9	10.11-15.69
	5	ND	ND	0.40	-0.84-1.64	0.9	-1.89-3.69
	4	D	ND	0.30	-0.94-1.54	0.7	-2.09-3.49
	5	ND	D	0.4	-0.84-1.64	0.9	-1.89-3.69
	73	D	ND	6.1	4.76-7.44	13.6	9.13-10.52
	78	D	NC	6.5	5.26-7.74	14.7	11.91-17.49
Mixed infection	1	D	D	0.08	-1.16-1.32	0.2	-2.79-2.79

Keys;

D= Deletion, ND = No Deletion, NC = Not Considered

N – Total number of sample = 1196

N– Total number of *P.falciparum* samples by PCR = 537

N= 83 (RDT False Negative Samples)

= 69 + 5+ 4 +5

Table 4.10 shows the plasma level of CPR, IFN-γ and IL-6 base different age groups.

The CRP has the highest plasma concentration among age group 0-10 with mean and standard deviation ($M \pm SD$) of 1.55±0.32 while the lowest is in the age group of 31-40 with $M \pm SD$ of 1.13±0.14. The p-value shows statistical significance among the different age groups.

The plasma concentration of IFN-Y has the highest concentration in the age group of 31-40 with M±SD OF 1805.76±18.19 and the lowest in the age group of ≥51with M±SD 1789.39±8.20. The p-value shows no statistical significance among the different age groups.

The plasma concentration of IL-6 has the highest concentration in the age group of 11-20 with a M±SD OF 175.23±10.82 and the lowest in the age group of 41-50 with M±SD of 152.90±7.04. The p-value shows statistical significance among the various age groups.

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Table 4.10: Age Distribution of CRP, IFN-γ and IL-6 Amongst Participants with PfHRP2 Gene deletion

Age Group	Participants Number	CRP (x) mg/dl	IFN-y (x) Pg/ml	IL-6 (x) Pg/ml
0-10	20	1.55±0.32	1792.59±26.53	171.95±14.89
11-20	16	1.38±0.42	1802.99±19.69	175.23±10.82
21-30	10	1.51±0.34	1795.48±17.09	164.43±25.27
31-40	8	1.13±0.14	1805.76±18.19	153.45±7.29
41-50	8	1.16±0.15	1795.76±8.50	152.90±7.04
≥51	11	1.23±0.17	1789.39±8.20	158.28±7.73
F		4.08	1.18	5.52
p-vaue		0.003	0.327	<0.001

CPR: C - reactive protein

IFN-γ: Interferon-Gamma

IL-6: Interleukin-6

PfHRP2: Plasmodium falciparum Histidine-Rich Protein2

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

This surveillance study provides evidence of PfHRP2 and PfHRP3 gene deletion in *P. falciparum* in Kaduna metropolis. The prevalence of PfHRP2 gene deletion found in the blood samples of 1196 participants was 6.1%, this prevalence is low compare to; Ghana (30%) Rwanda (25%), Eritrea (23%), but agreed with reports from Mali (5%), Equatorial (5.3%) Cameroon (5.8%), (Amoah *et al*, 2016, Kahama-Maro *et al*, 2011 and Koita *et al*, 2011,).

The samples of the participants were first diagnosed by RDT, and the negative samples obtained (694 RDT-negative) thereafter were further diagnosed by microscopy and PCR. Of these RDT negatives, microscopy diagnosed 83 samples to be positive (RDT false-negative) for malaria parasite and confirmed by PCR. This findings is in agreement with previous work by Eyong *et al* (2022)

The microscopy also identified 83 malaria parasites in the thick film. In the thin film, 5 non-falciparum species were identified out of the 83 RDT negative samples. Of these 83 RDT false-negatives samples, (6.9%) identified by PCR, 78 *P. falciparum, 2P. vivax*, 1*P. malariae 1P. ovale* and 1*P. falciparum/P. vivax.*

The WHO guideline considers a PfHRP2 deletion prevalence of 5% as a minimum threshold on change RDT type (Kahama-Maro *et al.*, 2011) or and if possible, confirm the result by another laboratory techniques, such microscopy.

With the prevalence of 6.1% PfHRP2 deletions in Kaduna metropolis, this serves as an alarm that it is now necessary to monitor the deletion of this gene across the different geographical location of the entire country in order to obtain a comprehensive data of deletions occurring with these genes. It is also important to take note that since this surveillance study was done in Kaduna, it becomes imperative for the study to be extended to cover the whole country.

With high prevalence of false-negative PfHRP2 and PfHRP3 deletion, this has significant implication for health. Once surveillance had been carried out and it is established that the threshold has been exceeded then it becomes imperative for an alternative RDT to be sourced and case management decision will have to be revised, with retraining in the use of the new RDTs. Further surveillance is necessary to investigate if such deletions must be carried out systematically and accurately (Kim *et al.*, 2008). If PfHRP2 deletions are found to be prevalent amongst symptomatic individual (the lower 95% CI is still above 5%), as is the case in countries like Eritrea in Africa, Brazil, Colombia and Peru in South America, malaria control programs will have to switch to RDT that do not only rely on PfHRP2 to detect *P.falciparum*. The WHO mandate 5% detection limit because it is around this point that the fraction of cases may be missed by the RDT (PfHRP2 as the target protein) due to non-HRP2 protein expression likely to be greater than that which would be missed by using less sensitive PLDH based RDT. Microscopical examination was of great importance in this study; all the 83 RDT false-negative samples that were positive by microscopy were confirmed with PCR diagnosis. The thick blood film was able detect the malaria parasite while the thin blood film was used to identify the species. Where microscopy is available for parasitological examination, this service should be strengthened to ensure parasite confirmation for malaria parasite (WHO, 2021).

The continuous and excessive use of PfHRP2 based RDT will enhance the selection of *P.falciparum* isolate when PfHRP2 deletion is present and identified as false negative.

Research reports have shown that detection PfHRP3 deletion may be signal to early warning sign for PfHRP2 gene deletion, therefore, it is highly essential to monitor the presence of parasite with PfHRP2 deletion to prevent RDT false-negative, as well as PfHRP3 deletion to act as early warning. This offers public health bodies ample time to step up surveillance effort and consider longer contingency plans (Ugah *et al.,* 2017 and Gamboa *et al.,* 2010).

The plasma CRP concentration result showed the highest mean and standard deviation of 1.55±0.32 mg/L in malaria parasitaemic participants of the age group 0-10 (positive by microscopy and PCR) amongst 73 RDT false-negative samples. This result is significant (P-0.003) compared to RDT true negatives (control samples), microscopy and PCR. The abnormally elevated plasma level of CRP in malaria infected participants showed that CRP is a positive biomarker for *P. falciparum* malaria and can also be used as an indicator of the disease in addition to febrile symptoms. This finding agrees with Emmanuel *et al*, 2019. However, there is no difference in CRP levels in participants' blood samples between those with intact PfHRP2 gene and those with PfHRP2 gene deletion. CRP values also vary in malaria among different age groups, research indicates that CRP levels in young children with malaria indicating great inflammatory response (research. net). CRP correlates positively with age in *P. falciparum* malaria (p=0.008). In another finding, CRP levels were not significantly affected (p=0.766) or gender (p=0.111) in severe malaria (Abdelwahab *et al*, 2022)

The plasma level of pro—inflammatory cytokines; interferon gamma (IFN-γ) and interleukin 6 (IL-6) were also evaluated. The M±SD plasma levels for IFN-γ and IL-6 in 73 participants (RDT false-negative) with malaria parasite (positive by microscopy and PCR) was 1805.76±18.19 mg/L and 175.23±10.82 mg/L respectively. However, in the control participants' samples, RDT, microscopy and PCR negative, the serum levels of the IFN-y and IL-6 were reference range with mean plasma level of 113.86±51 and 65.78±33respectively. This finding indicates no statistical significance for IFN-y(P-0.327) and IL-6 is statistically significance ($p<0.001$) difference between infected and uninfected participants. This result is in agrees with finding of Ezeokoli et al, 2023. Also this finding does not show any difference in these pro-inflammatory cytokines between intact PfHRP2 gene and those with PfHRP2 gene deletion.

Previous study indicate that it is the *P. falciparum* parasite that lack the gene (PfHRP2) and not the blood sample, therefore, the inflammatory process and response in malaria infection is not the cause of the PfHRP2 gene deletion. This finding is in agreement with the report of Gupta *et al*, 2017 that PfHRP2 gene deletion is caused by the inability of the *P. falciparum* to produce PfHRP2 gene.

5.2 Findings

The findings from this study are as follows:

1. The molecular analysis confirms the presence of PfHRP2 and PfHRP3 genes; it also confirms that there were deletions in PfHRP2 and PfHRP3 genes.
2. The molecular analysis confirms 83 RDT-negative samples were indeed false-negative. Of these, 69 had deletion in both PfHRP2 and PfHRP3, 4 had deletions in PfHRP2 gene only, 5 had deletion in PfHRP3 gene only, 5 had no deletion in both PfHRP2 and PfHRP3 gene and 1 had no PfHRP2/3 gene. However the epidemiological findings show PfHRP2 gene deletion was detected in 73 RDT-negative samples (RDT false-negative) with prevalence rate of 6.1% amongst 1196 participants.
3. The findings also indicate that PfHRP2 gene deletion was the cause of RDT false negative results in 73 samples (RDT-Negative).

4. This surveillance study also indicates there is no significant difference ($p<0.001$) in Plasma level of CRP, IFN- and IL-6 of blood samples of participants with intact PfHRP2 gene and those with PfHRP2 gene deletion.

5.3 Conclusion

This surveillance highlights the concern of the prevalence of PfHRP2 gene deletion and its implication on the use of rapid diagnostic tests (RDT) for malaria diagnosis. *P. falciparum*

isolate with PfHRP2 gene deletion were present in the 73 RDT negative samples and this is the cause of RDT false-negative result in Kaduna.

Although, this finding is confined to Kaduna metropolis, this result is too limited to reconsider the efficacy of RDT, however these deletions (PfHRP2) represent a serious threat to malaria diagnosis since a lack of available quality test may result to lack of adequate treatment. This may mean uncontrolled infection (due to false-negative result) and this might increase the rate of malaria morbidity and mortality. The overall implication of these deletions will be more severe in areas with low prevalence of malaria particularly in other regions that are close to malaria control and eradication.

This review also highlighted the need for better characterization of the threat posed by these deletions. To this end, a standardized methodology for all studies could play a key role in increasing the understanding of these deletions, their transmission dynamics, modifiers and associated effects thereby allowing comparism between different geographical regions of Nigeria in terms of epidemiological variables.

5.4 Recommendations

Findings from this surveillance studies, it is recommended that regular and systematic surveillance of PfHRP2 gene deletions should be adopted as part of national guidelines for malaria control policy.

It is highly recommended for malaria diagnostic strategies to combine two or more target protein (antigen) to evaluation of diagnostic quality of the RDT.

It is also recommended that for malaria diagnosis in malaria endemic areas, microscopy, a gold standard, should not be ignored. Microscopy had been neglected in many places due to the advent of RDTs. Though, RDT method is cheaper and gives malaria diagnosis no less than 30 minutes, the availability of high-quality rapid test in combination with quality microscopy would enable diagnostic and reference centres to offer better healthcare to the population.

5.5 Contribution to Knowledge

The contributions of these surveillance research findings to knowledge include:

1. The study contributes to the understanding of prevalence of PfHRP2 gene deletion across different age groups which will improve knowledge on malaria diagnosis.

2. The study contributes to the understanding of protein deletion and diagnostics. Monitoring diagnostic accuracy of PfHRP2 gene deletion by immunochromatographic method can affect the accuracy of rapid diagnostic test (RDT).

3. The data obtained from this study on based on evidence of existence of PfHRP2 deletion can enable inform decision on diagnostic test selection and RDT deployment.

4. The understanding of the prevalence of the PfHRP2 deletion can help policy makers to develop effective treatment strategies.

5. By monitoring PfHRP2 deletions, researchers can understand the dynamics of malaria transmission and develop targeted interventions.

6. Measuring serum levels of IFN-γ, IL-6 and CRP can contribute to the understanding of immunological pathogenesis, molecular mechanisms and as a biomarker for malaria infection and disease progression.

7. Profiling of cytokines, especially IFN-γ and IL-6 and acute phase protein, CRP contributes to the understanding of immune response to malaria infection. Elevated levels of IFN-γ, IL-6 and CRP may indicate activation and inflammatory response to malaria infection.

8. The study will also contribute to knowledge in advances made in immunological and molecular epidemiology, which aims to increase the understanding of the distribution and determinants of infectious diseases (malaria) diagnosis at the molecular level**.**

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APPENDIX

3.5.6 Materials Required But Not Provided

I. Stop watch (Timer)

II. Disposable hand gloves

III. Pencil marker

IV. Biosafety sharps container

V. Biohazard waste container

VI. Sterile gauze or cotton

VII. Methylated spirit

3.5.7 Precautions

II. Laboratory coat, hand gloves and nose mask were worn

III. The insert literature instructions was carefully followed to get accurate result

IV. The device was taken out from the sealed pouch and test carried out immediately

V. The test Kit was stored at room temperature mentioned on the kit in a dry environment

VI. No expired kit was used for the diagnosis

VII. Test kit were never reused for the second time

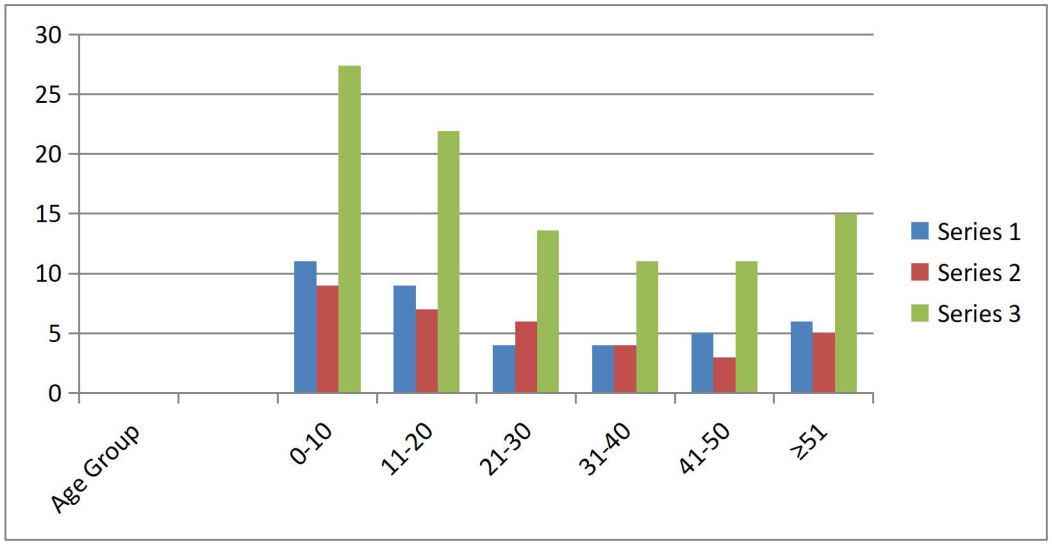
VIII. There was no mouth pipetting

IX. Only buffer supplied by the manufacturer was used

X. All samples and test kits were disposed according to GLP

Figure 4.9 Bar chart showing demographic distribution of prevalence of PfHRP2 gene deletion. Of these samples (73) with PfHRP2gene deletions; 21 (27.7%), 16 (20.7%), 11 (14.2%), 8 (10.3%), 9 (12.9%) and 11 (14.2%) samples in the respective age groups (0-1) and above. This surveillance findings shows that the age group (0-10) and (11-20) have highest and combined (48%) episode pfhhrp2 gene deletions amongst the various age groups. The age group (31-40) has the least 8 (10.3%) episode of gene deletion. Based on

gender, there was higher gene deletion in males 43(55.8%) in male than in females (34, 44.2%)



Bar Chart Demographic Distribution of Prevalence of PfHRP2 Gene Deletion

Keys;

Blue colour = male

Maroon colour = female

Green colour = percentage prevalence

Table 4.7 shows data that was processed and used for statistical analysis

83 Samples	**N(1196)**	**(95 % CI)**	**N=537**	**(95 % CI)**
69	5.77	4.56 - 7.04	12.85	10.11 – 15.69
5	0.42	-0.48 – 1.64	0.93	-1.89 – 3.69
4	0.33	-0.96 – 1.54	0.74	-1.09 – 3.49
5	0.42	-0.48 – 1.64	0.93	-1.89 – 3.69
78	6.52	5.26 – 7.74	14.53	11.91 – 17.49
1	0.08	-1.16 – 1.32	0.19	-2.79 – 2.79

Keys:

N = Total participants, **n** = Sample, **LL** = Lower Limit of Confidence interval, **UL** = Upper Limit of Confidence interval, **SE** = Standard Error

Table 4.7 shows data that was processed and used for statistical analysis. The total number of samples N, the n which is the various subset of N, lower Limit (LL) and upper limit (UL), and confidence interval (CI).

MINISTRY OF HEALTH
KADUNA STATE, NIGERIA

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NOTICE OF EXPEDITED REVIEW AND APPROVAL

"PLASMODIUM FALCIPARUM HISTIDINE RICH-PROTEIN 2 GENE DELETION AMONGST SYMPTOMATIC MALARIA PARASITAEMIC PATIENTS IN KADUNA METROPLIS".

Name of Principal Investigator:	Usman Itakure Abdulkadir
Address of Principal Investigator:	Dept. of Medical Laboratory Science School of Basic Medical Sciences University of Benin Benin City
Date of receipt of Application:	18th juiy,2023
Date of Ethical Approval:	24th July, 2023
Date of Expiry of Approval:	24th July, 2024

This is to inform you that the Research described in the submitted Protocol, the Consent Forms,advertisements and other participant information have been reviewed and given Expedited approval by the Health Research Ethics Committee (HREC) of Kaduna State Ministry of Health.

If there is delay in starting the research or any need for correction, inform the HREC secretariat so that the dates of approval or other corrections can be effected accordingly.

However, Researcher is kindly requested to submit a copy of his/her findings to the State Ministry of Health,please.

Dr.Sunday Joseph
For: Chairman (HREC)

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