# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

Malaria is caused by *Plasmodium* parasites. Anopheles mosquitoes called malaria vectors spreads the parasites to people through bites. Five species of the parasites are known to cause malaria in human. Two of these species, namely *P.falciparum* and *P. vivax* pose the greatest threat. *P.falciparum* is the most prevalent malaria parasite on the African region. It accounts for most of the deaths due to malaria. Outside sub Saharan Africa, *P.vivax* is the common malaria parasite known (WHO, 2016)

Malaria is still the world's most crucial tropical parasitic disease with about 40% of the world's population at risk of infection (Garcia *et al.*, 2010)Although significant progress has been achieved in recent years at reducing malaria-relatedmorbidity and mortality due to global malaria control measures, an estimated 216 million cases still occurred in 2010 leading to about 655 000 deaths (WHO, 2011). There were further reduction in 2015 to 429 000 malaria deaths worldwide. 92 % of these deaths occurred in African continent, followed by South-East Asia with 6 % and the Eastern Mediterranean region with 2 % (WHO, 2016). Malaria incidence rates globally and in the African region fell by 21 % between 2010 and 2015. Also at the same time, malaria mortality fell by 29 % globally and 31 % in the African Region.

Under 5 children are the most prone to malaria illness, infection and death. Malaria killed an estimated 303 000 under-five globally, including 292 000 in the African region (WHO, 2016). Progress has been made so far between 2010 and 2015. Within this time, mortality rate had been reduced by 35 %, though malaria is still the main killer of under-fives.

The main key to prevent and reduce malaria transmission is through vector control. Insecticides treated mosquito nets (ITNs) and indoor residual spraying (IRS) are the two forms of vector control that has demonstrated to be effective. ITNs are the main thrust of malaria prevention efforts, especially in sub-Saharan Africa (WHO, 2016). In the last past five years, the use of treated mosquito nets has improved. In 2015, an estimated 53 % of the population at risk slept under a treated net compared to 30 % in 2010(WHO, 2016). IRS is used by national malaria programmes in targeted areas. One hundred and six (106) million people globally were protected by IRS, 49 million people in African inclusive.

Currently, Artemisinin – based combination therapies (ACT) remain the drug of choice in treating malaria. It's been observed to be effective against the most prevalent and lethal malaria parasite affecting humans (WHO, 2016). The number of ACT treatment courses procured from manufacturers

Increased from 187 million in 2010 to a peak of 393 million. This letter went down to 311 million in 2015 (WHO, 2016). Other drugs like chloroquine had been withdrawn in 2005 to check resistance due to drug pressure.

Malaria in pregnancy exposes the mother, her foetus and the new-born child to substantial risks. Over time, the number of women in Africa who receive intermittent preventive treatment in pregnancy (IPTp) for malaria had increased, though coverage remain below national targets (WHO, 2016). IPTp is given to pregnant women at planned antenatal care visits after the first trimester. It helps to prevent maternal death, anaemia and low birth weight, a major cause of infant mortality. Between 2010 and 2015, there was a five-fold increase in the delivery of 3 or more doses of IPTp in 20 of the 36 countries that have adopted WHO's IPTp policy – from 6% coverage in 2010 to 31% coverage in 2015 (WHO, 2016).

## 1.2 Statement of Problem

Malaria is still the world's most crucial tropical parasitic disease with about 40% of the world's population at risk of infection (Garcia *et al*., 2010). World Health Organization estimated in December, 2016 that there were 212 million cases of malaria in 2015 and 429 000 deaths annually. Amongst the four species known to cause malaria in human, *P.falciparum* is the most virulent that is highly associated with life threatening severe cases of malaria. *P.vivax* usually causes repeated relapses, rare life threatening complications (White *et al.*,2003) and low – birth – weight infants of infected mothers which increases the risk of death in new-born (Nosten *et al.*, 1999)

Malaria enforce substantial costs to both individuals and government. Costs to individuals and their families include purchase of drugs for treating malaria at home; expenses for travel to, and treatment at, dispensaries and clinics; lost days of work; absence from school; expenses for preventive measures; expenses for burial in case of deaths (CDC, 2016). Costs to governments include maintenance, supply and staffing of health facilities; purchase of drugs and supplies; public health interventions against malaria, such as insecticide spraying or distribution of insecticide-treated bed nets; lost days of work with resulting loss of income; and lost opportunities for joint economic ventures and tourism. Direct costs (for example, illness, treatment, premature death) have been estimated to be at least US$ 12 billion per year (CDC, 2016).

Progress in malaria control is been threatened in many countries due to rapid development and spread of antimalarial drug resistance. Olukosi*et al*., in 2014 reported persistence of chloroquine resistance maker in Lagos state, Nigeria(Olukosi*et al.*, 2014). To date, parasite resistance to artemisinin has been detected in some countries. Though not yet reported in some African sub regions, but there are indications that ACT would soon be compromised by

drug resistance. Mosquito resistance to insecticides is another growing concern in many countries presently.

Improper diagnosis of malaria infections is another big challenge to effective treatment of the disease. Many laboratory personnel lacked the capacity to correctly identify the parasite. Some are conversant with *P.falciparum* to the point that other species of malaria are also termed *P.falciparum*. There are many cases of mixed infections in circulation, but use of microscopy alone has limited correct detection of these parasites, in qualitative and quantitative approach.

### 1.3 Aim and Objectives of the Study

**Aim:**

The study was designed to characterize the species of *Plasmodium* in Nnewi, Southeast Nigeria and assess the genetic diversity and prevalence of antimalarial resistance gene in circulation in the region (a baseline).

**Objectives:**

1. To determine the parasite densities of malaria asymptomatic cases in Nnewi, southeast Nigeria
2. To evaluate the usage of mosquito bed nets and other vector repellents
3. To molecularly assess for *Plasmodium* species in all the samples
4. To carry out Genetic diversity studies on the *P.falciparum* species using relevant markers
5. To determine the prevalence of drug resistance genes on the isolated *P.falciparum*

### 1.4 Justification/ Significance of the Study

Malaria has received global attentions in the past decades and in recent times. The present decline in the morbidity and mortality rates due to malaria infection is the resultant effect of the comprehensive attention from the global society (WHO, 2016). Though, more than 90 % cases of malaria are housed in African, a lot of researches on the same disease were done bycolleagues in Europe, America and UK. There are deep gaps in the knowledge of malaria parasite in Nigeria. Currently, there are limited data on the nature, character and genetic diversity of the malaria parasites in the country. Few persons in the past had attempted to provide baseline data on the molecular nature of the parasite in southwest Nigeria. Happi*et al.,* in 2004 published a report on 'Molecular analysis of *Plasmodium falciparum* recrudescent malaria infection in children treated with chloroquine in Ibadan, southwest Nigeria'. Oyebola*et al.,* in 2014 published 'Genetic diversity and complexity of *Plasmodium falciparum* Infections in Lagos, Nigeria. In Northern Nigeria, Oyedeji, *et al.,* in 2013 published a work on 'Genetic diversity of *Plasmodium falciparum*isolates from Naturally Infected Children in North – Central Nigeria Using the Merozoite Surface Protein -2 as Molecular Marker. As at the time of this report, there is no data from Nnewi, southeast Nigeria. Hence, the present work aims to provide baseline data on the *P. falciparum* antigenic makers circulating in Nnewi, Southeast Nigeria.

# CHAPTER TWO
# LITERATURE REVIEW

## 2.1. Definition and Reports

Malaria is a complex disease that varies widely in epidemiology and clinical manifestation in different parts of the world. It's caused by organisms that belong to the protozoan group namely *Plasmodium*. It is a severe and potential fatal disease which poses a major health threat to humans since the new stone age revolution around 10,000 years ago (Tishkoff*et al*., 2001). It remains an undisputable public health concern in countries where transmission occurs regularly, as well as in places where transmission is low resulting from control and elimination (Bloland, 2001). Malaria is caused by five species of *Plasmodium* that infect humans (*Plasmodiumfalciparum*, *Plasmodiumvivax*, *P. ovale*, *P.malariae* and *P. knowlesi)* and is transmitted exclusively by bite of infected female anopheles mosquitoes. The intensity of transmission depends on factors related to the parasite, the vector, the human host and the environment (WHO, 2013).

Despite many efforts to eliminate malaria, it has continued to be of huge impact to human health and is still a risk for around 40% of the world population (WHO, 2013). Mortality is high in people not protected sufficiently by an acquired immunity such as young children, pregnant women and especially in migrants or travelers originating from regions where malaria is not found (WHO, 2016)

According to WHO, a child dies of malaria every 30 seconds and nearly 250 million cases of this disease causing one million deaths (Lorenz *et al.*, 2014). According to the present report, malaria had declined since 2010 and 2015. Annual death is currently estimated around 429, 000(WHO, 2016)

Although malaria is not listed as a neglected disease by WHO, there has been an effective drug development adopted by governmental, private and public funding as well as WHO, Bill and Melinda Gates foundation. The global health plans have shown strong political and social willingness to strengthen the development of anti-malaria measures including a malaria vaccine (WHO, 2016).

### 2.2 *Plasmodium* life cycle.

Malaria is caused by monocellular organisms that belong to the genus *Plasmodium* with its species *Plasmodiumfalciparum*, *Plasmodium vivax*, *P. ovale*, *P.malariae* and in some cases *P. knowlesi*in Asia. *Plasmodium* life cycle revolves around two different hosts, humans and female anopheles mosquito bite (WHO, 2013). A person becomes infected after being bitten by an infected female Anopheles mosquito. When a mosquito bites, it injects its saliva that contains parasites sporozoites into the person's blood stream. The sporozoites are transported through the circulation to the liver and invade the hepatocytes. Here the parasite hide from the immune system, undergo numerous divisions, multiply in numbers and pass through schizonts and merozoites developmental stages. After about 1 to 2 weeks a person is bitten by an infected female mosquito, the multiplying parasites causes rupture of the infected liver cells. In the course of rupture of the hepatocytes, the merozoites will be released into the vesicles that circulates into the blood stream. The merozoites invade the red blood cells, performs multiple divisions to yield trophozoites and schizonts which are released into the blood stream upon rupture. The blood cell cycle repeats and is the basis for regular fever symptoms. Few merozoites eventually develop to gametocytes that get ingested by a mosquito. In the mosquito gut, *Plasmodium* gametocytes ingested with human blood develop into gametes. The gametes fuse to form a zygote that gets motile (ookinate) and invades the stomach lining of the mosquito as oocysts.

The oocysts develop into sporozoites that evades the guts and enter the mosquito's salivary gland ready to infect humans upon feeding as shown in the figure below.

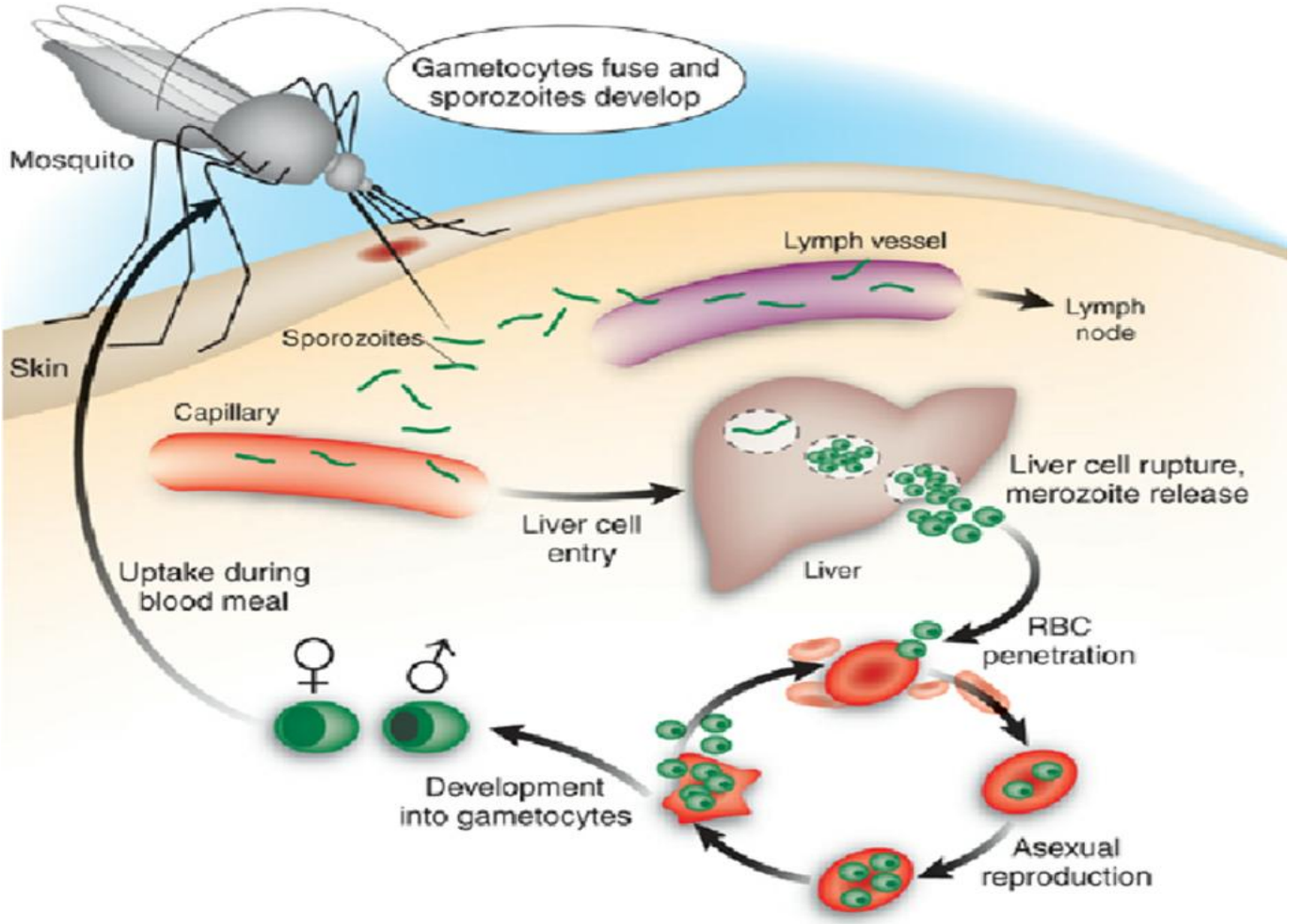



**Figure 1: The *Plasmodium* life cycle**

**Source:** Jones and Good, (2006)

## 2.3 Epidemiology of malaria

It has been reported that malaria is prevalent in tropical and subtropical regions of the world, especially in Africa, south of Sahara, south East Asia and the forest fringe zones in South America (WHO, 2012). In 2008, WHO had a record of 250 million cases each year world wide of which more than 86% occur in sub Saharan Africa (Adebayo *et al*., 2012).

In 2010, out of the 219 million cases reported by WHO, 660,000 cases of clinical malaria was estimated to cause death globally (WHO, 2012). Although Murray *et al*., (2010) reported calculated cases of death due to malaria to be around 1.2 million in 2010. Sub Saharan Africa accounts for the main burden of overall malaria incidences (80% of the cases), overall malaria death (91% of the cases) and deaths in the age group of 0-5 years (86% of the cases) (WHO, 2012). According to WHO, 98% of all the reported cases are mostly caused by *P. falciparum* (WHO, 2012).

In Asia, Latin- America and some part of Africa, *P. vivax* has been implicated to cause rarely fatal malaria outbreak. *P.vivax* can form dormant liver stages known as hypnozoites that can get active after months and years of infecting mosquito bite (CDC, 2013).

Malaria incidence and death cases reported in Africa were focused in 13 and 15 countries respectively and have been connected to poverty and people living in rural areas (WHO, 2013).

In 2015, WHO reported an estimated number of 438,000 people that died of malaria, with over 90% of these deaths occurring in sub-saharan Africa, while others occurred in south East Asia and South American (WHO, 2015). The report recorded that most of these deaths occur in African children younger than 5 years. Worldwide the number of new episodes of clinical malaria in 2015 is estimated to be around 214 million (WHO, 2015).

Areas of stable or high moderate transmission are places where populations are continuously exposed to a fairly constant rate of malaria. In these areas, immunity is developed during childhood while adults and adolescents are partially immune, although they may have a few parasites in their blood (Adebayo *et al*., 2012). In areas of unstable or low transmission, the population is not exposed to malaria very often. In these areas, the incidence is seasonal (such as in the rainy season) and as a result of these low levels of malaria infection, the population develops little or no immunity.

*P. vivax* is dominant *Plasmodium* species in endemic areas outside Sub-Saharan Africa (WHO. 2016). In endemic areas, malaria parasite transmission may occur throughout the year often with seasonal increase in intensity of rainfall (Reiter, 2008). Transmission intensity varies as a function of biting and survival rate of the mosquito vector, density, temperature, humidity as well vector control measures (Boccolini*et al*., 2012). Transmission of malaria may vary within a country because of difference in ecological and climatic factors, which influences the abundance of vector breeding sites and survival of mosquitoes.

The dominant clinical signs of uncomplicated malaria are fever that can occur intermittently and has given malaria the name 'remittent fever'. Malaria quartana is caused by *P. malariae* and *P. ovale*, resulting in fever that repeats every 48hours. Fever caused by *P. falciparum* has an irregular occurrence and does not follow the remittent fever cycle (Bezirtzoglou*et al*., 2011).

Further symptoms of malaria are headache, chills and vomiting, as well as speech difficulties, deafness or blindness. Non –severe malaria is well curable based on correct diagnosis and with appropriate therapy as recommended (WHO, 2016).

**2.4 Unmet needs beyond current malaria prevention and treatment practice**

Today a range of options exist to prevent mosquito bites, to implement a medical prophylaxis and to apply diverse treatment options in case a malaria infection occurred. Ways to mechanically prevent mosquito bites and thus a malaria infection are to use insecticide treated bed nets (ITNs), especially long lasting insecticide treated bed nets (LLIN), indoor insecticide spraying (also called "indoor residual spraying", IRS) or mosquito control in the natural habitat (WHO, 2012). The bed nets can be used effectively over three years before they need to be exchanged.

Moreover, several studies indicate emergent resistances against the insecticides, as in Mozambique, Ghana and Uganda (Hunt *et al*., 2011; Morgan *et al*., 2010). Similarly to emerging insecticide resistances, resistances against larvicides used in land control programmes has been observed. This indicates that existing measures have a high risk to be ineffective in the near future and new compounds are required (WHO, 2011).

Main therapies of clinical malaria include monotherapy with quinines and artemisinin combination therapies (ACT) as recommended by the WHO (WHO, 2015). This however requires that quality drugs are available globally. Instead, ACT availability is obstructed by supply constraints, quality issues and counterfeits. In South-East Asia, for example, it was estimated that around 30-35 % of ACTs are counterfeits (Ambroise, 2012).

An easily manageable disease, with prevention measures functioning on a high quality and long term level, would likely not result into efforts to develop a vaccine for global implementation

The under-coverage of measures globally and the need to replace currently used drugs and chemicals due to emerging resistances can be understood as a gap in malaria control globally and hence the need for better solutions.

Preventive vaccines are known to have a good safety profile that is better than therapeutic treatments and they are judged to be the most efficient tools for promoting individual and public health (Andre *et al.*, 2008) Main characteristics are that preventive vaccines can provide a durable prevention against a disease and accordingly prevent burden that would occur through a clinical manifestation and the recommended treatment with its side effects, required treatment schedules and costs. Although smallpox is so far the only disease that has been eradicated through the implementation of vaccines, local elimination of other diseases like measles or mumps is reported. There is estimation that almost 6 million deaths per year can be prevented by vaccines (Andre *et al*., 2008).

It has been established that a preventive malaria vaccine could be a potent, effective and affordable tool to reduce malaria incidence and mortality and leading to huge savings of costs currently correlated to malaria (Malaria Vaccine Initiative, 2013). Immune mechanisms are part of the natural defence strategy and can be raised artificially in animal models and in human beings, making it reasonable and feasible to develop a protective vaccine against malaria (Andre *et al*., 2008).

**2.5 Malaria vaccine**

Resistance to antimalarial drugs has often threatened malaria elimination efforts and historically has led to short term resurgence of malaria incidence and deaths. In response to an effective and long lasting solution to eliminate malaria, J. Gordon Thomson addressed the Royal Society of Tropical Medicine and Hygiene on the topic of Immunity in Malaria (Sardá*et al.*, 2009). This paved way to subsequent researches on malaria parasite immunogen. Fifty years later, from the time he addressed Royal society of Tropical Medicine, the first gene encoding a malaria immunogen, the circumsporozoite protein (csp) was molecularly cloned and sequenced(Sardá*et al*., 2009). This was followed by identification and cloning of

immunogens of the parasite infective stages, merozoite surface protein 1(MSP1) by Dame and colleagues, (Dame *et al*., 1984). Evidence from studies that supports the existence of naturally acquired immunity formed the basis for development of potential malaria vaccines that target immunogens of different infective stages in the parasite life cycle (Sharma and Pathak, 2008)

With around 40% of the world population at risks, malaria vaccine is a matter of Global interest. Malaria vaccines under development belong to the group of vaccines that causes active immunization through stimulation of an immune response by vaccinated individual. Scientifically, it was seen that immune mechanism are part of the natural defence strategy and therefore can be raised artificially in human and animal models, making it feasible to develop a protective vaccine against malaria (WHO, 2016).

### 2.5.1 Prospects on malaria vaccine

WHO, through malaria vaccine Advisory committee and Initiative for Vaccine Research (IVR), has established a malaria road map to strengthen the development of a malaria vaccine. This road map has been effective by defining goals related to timelines, safety and efficacy of a quality malaria vaccine based on international standards. To accomplish this, efforts and supports are extensively made by foundations and governmental organizations as well as vaccines and clinical trial initiatives (Malaria Vaccine Technology Road Map, 2006)

### 2.5.2 Asexual/blood stage Vaccine

One of the approaches for the development of a malaria vaccine is to target immune responses against the asexual stage of the parasite (blood stage). Blood stage vaccines targeting the asexual stages are aimed to prevent multiplication of merozoites in the blood cycles and thus should reduce overall parasite burden resulting into less severe and less frequent clinical disease targeting ideally complete prevention of clinical disease. The

underlying principle for this approach according to Ballou *et al*., (2004) is based on the observations that

a) Maternal antibodies passively transferred to the fetus may provide a window of protection against clinical malaria,

b) Following repeated attacks of malaria, a majority of infected individuals living in endemic areas acquire the ability to control parasite replication to levels below those that result in clinical disease, and

c) Hyper immune globulin prepared from the sera of individuals chronically infected with malaria can eliminate circulating parasites from*P.falciparum* infected individuals.

The principal target of current asexual stage vaccine development is the merozoite, the stage that is originally released from the infected hepatocyte and rapidly invades and replicates in circulating red blood cells. The antibodies are produced against the surface proteins of merozoite actively block invasion of the red blood cells by the Merozoite. The most studied antigens include,

a) Merozoite surface protein 1 (MSP-1),

b) MSP-2,

c) MSP-3,

d) Apical membrane antigen 1 (AMA-1).

These molecules are transiently accessible to circulating antibodies. Antibodies to these molecules are reported to block invasion of merozoites, except for MSP-3, in which they trigger a monocyte-mediated effect. MSP-1, AMA-1, and MSP-3 have been produced as candidate malaria vaccines and have been shown to protect non-human primates from uncontrolled asexual stage parasitaemia when administered with Freund's complete adjuvant.

Moreover antibodies to MSP-3 can reproducibly transfer protection in a new mouse model of *P. falciparum*malaria (Ballou *et al*., 2004)

The merozoite surface protein 1 (MSP-1) is involved in the merozoite invasion of the erythrocyte and intracellular merozoite development. First attempts with a MSP-1containing subunit vaccine were poorly immunogenic, caused hypersensitivity reactions. (Otsyula*et al*., 2013).

The AMA-1 protein is involved in the hepatocyte invasion by the sporozoite, but mainly known to inhibit merozoite invasion of the red blood cell, and is therefore classified as a blood stage vaccine. AMA-1 based subunit vaccines have achieved protection against malaria challenge in animal models and anti-AMA-1 antibodies have been showed to inhibit *P. falciparum* growth in-vitro (Bruder*et al*., 2010).

The major problem in the development of a malaria vaccine is the marked parasite strain variability associated with many blood stage antigens that require the selection of targets that relatively combine two or more antigens or allelic forms of a single protein (Valupadasu and Mateti, 2012). Blood stages of *Plasmodium* are characterized by a high proportion of gene polymorphisms. This gene polymorphism prevents complete efficacy of blood stage vaccines and may conversely promote further escape mechanisms by the parasite resulting potentially into *Plasmodium* forms that are more pathogenic than the existing forms (Riley and Stewart, 2013).

### 2.5.3 Malaria vaccine candidate antigens:

Bearing in mind the whole parasite life cycle, the summary of the six target antigens for a malaria vaccine is presented in the table below.

Table 1: Main vaccine candidates from different phases of *Plasmodium* life cycle

| Targets | Candidate antigens |
|---|---|
| Sporozoite | Circumsporozoite protein (CSP)<br>Thrombospondin-related adhesive protein (TRAP)<br>Sporozoite and liver-stage antigen (SALSA)<br>Sporozoite threonine- and asparagine-rich protein (STARP) |
| Liver stage | CSP<br>Liver-stage antigen (LSA)-1 and -3<br>SALSA<br>STARP |
| Merozoite | Merozoite surface protein (MSP)-1, -2, -3, -4 and -5<br>Erythrocyte-binding antigen (EBA)-175<br>Apical membrane antigen (AMA)-1<br>Rhoptry-associated protein (RAP)-1 and -2<br>Acidic-basic repeat antigen (ABRA)<br>Duffy-binding protein (DBP) (*Plasmodium vivax*) |
| Blood stage | Ring erythrocyte surface antigen (RESA)<br>Serine-rich protein (SERP)<br>Erythrocyte membrane protein (EMP)-1, -2 and -3<br>Glutamate-rich protein (GLURP) |
| Toxins | Glycosilphosphatidylinositol (GPI) |
| Sexual stages | Ps25, Ps28, Ps48/45 and Ps230 |

**Source**: Carvalho*et al*., 2002

### 2.5.3.1 Circumsporozoiteproteins (CSP)

The circumsporozoite (CS) protein is a cell surface protein of the sporozoite, the stage of the life cycle of malaria parasites (*Plasmodium*species*)* that infects the vertebrate host.

The experiments by Nussenzweig, and Vanderberg which systematically established that irradiated sporozoites do confer protection to the respective vertebrate host led to an immediate hunt for protective antigens of the sporozoite, and the circumsporozoiteprotein (CSP) was identified (Richie *et al*., 2015).

Circumsporozoite gene from the simian malaria parasite, *P. knowlesi* H-strain was the first malaria gene to be cloned(Sharma and Pathak, 2008).

The CS protein covers the entire surface of the mature sporozoite (Hughes, 1991). The CS gene, is present in a single copy per haploid genome, lacks introns and encodes a protein which varies in length between species but is usually around 400 amino acid residues long (Hughes, 1991). The CS gene can be divided into three regions namely:

-The 5’ non-repeat region (5’NR);

- A central repeat region, consisting of one or two short motifs (4- 12 codons in length) repeated in tandem numerous times; and

-The 3’nonrepeat (3’NR) region.

Suggestion from a number of different host and parasite species indicates that the repeat region is the target of antibodies against the CS protein ((Ballou *et al*., 2004). The most promising CS candidates include immunodominant B cell epitopes present in the central repeat (NANPx) region (several repeats of asparagine-alanine-asparagine proline peptides) and T cell epitopes from the C terminal portion of the molecule (Stoute*et al*., 1997)

### 2.5.3.2 Thrombospondin-related adhesive Protein (TRAP):

Thrombospondin-related adhesive protein (TRAP), (Table 1), is a protein mobilized from microneme to the surface of sporozoite(Rogers *et al*., 1992). TRAP contains a hydrophobic N-terminal peptide (domain I), an integrin-like magnesium binding A domain (domain II), thrombospondin type I repeats (domain III), an acidic proline/asparagine-rich region (domain IV), hydrophobic transmembrane domain (domain V) and a cytoplasmic tail (domain VI) (Kosuwin*et al*., 2014). TRAP has been shown to mediate gliding motility and invasion processes of malarial sporozoites into vertebrate's hepatocyte and mosquito's salivary gland (Kosuwin*et al*., 2014). Two extracellular portions of malarial TRAP, the von Willebrand A-domain and the thrombospondin repeats located in domains II and IV, respectively, are crucial for initial host cell adherence and stabilization of adhesion/de adhesion during gliding mobility of sporozoites (Matuschewski*et al*., 2002). Crystal structure analyses spanning the von Willebrand A-domain and the thrombospondin repeats of PvTRAP and PfTRAP reveal two conformational states, open and closed structures. Such structural phase transition is possibly responsible for 'stick-and-slip' or gliding motility of sporozoites through the actomyosin motility apparatus(Song *et al*., 2012)

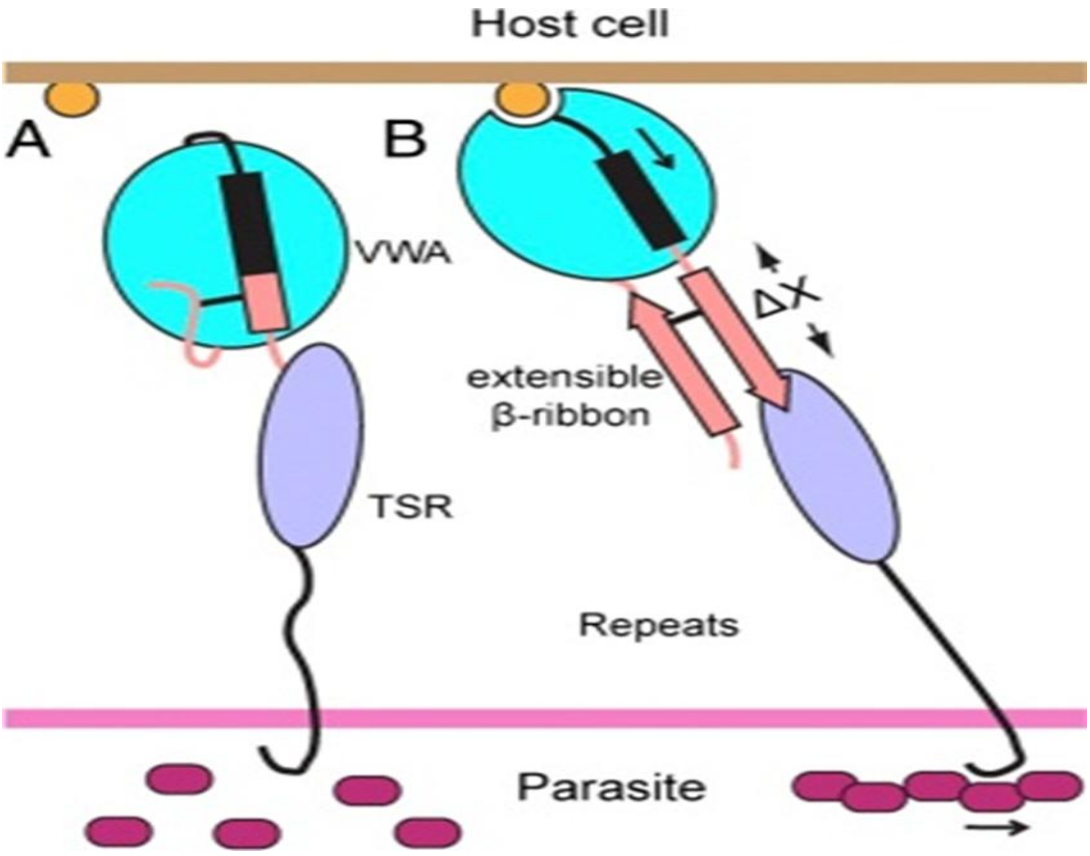



**Figure 2: Model of Thrombospondin-related adhesive protein(TRAP) activation**.

**Source**; (*Song et al*., 2012)

(A) In the absence of ligand and tensile force, the closed conformation with a flexible orientation between the VWA and TSR domains is favoured.

(B) When an immobilized ligand binds to TRAP through the MIDAS, the tensile force exerted by the parasite actomyosin apparatus will be resisted. TRAP will then be elongated and straightened along the force vector. The elongational force is on pathway with TRAP extension through activation of the VWA domain and formation of the extensible β ribbon. Hence the extended open conformation with putative high-affinity is favoured.

At the liver stage of malaria infection by *P. falciparum*, TRAP is processed through the endogenous pathway, and the resulting antigenic peptide fragments are presented by HLA

class I molecules to T cell receptors (TCRs) on cytotoxic T lymphocytes (CTLs) (Ohashi*et al*., 2014). Earlier studies which pointed out that a wide range of pre-erythrocytic TRAP antigens are targeted by naturally acquired CTLs (Doolan*et al*., 1997), together with the fact that TRAP has CTL epitopes, presents TRAP molecule as a major target of the human immune response to pre-erythrocytic stages of malaria due to *P. falciparum*.

The roles of anti-TRAP antibodies in partial Protective immunity against malaria have been demonstrated in African endemic areas (Ayieko *et al*., 2017; John *et al*., 2008).

#### 2.5.3.3 Erythrocyte binding protein (EBA)

Erythrocyte-binding antigen 175 (EBA-175) is the best-characterized *P. falciparum* invasion ligand, reported to recognize glycophorin A on the surface of erythrocytes (Chiu *et al*., 2016). Its protein structure comprises 6 extracellular regions, where region II contains Duffy binding–like domains involved in the binding to glycophorin A.

#### 2.5.3.4Merozoite surface protein (MSPs)

Early electron microscope images of *Plasmodium*merozoites revealed that they were covered in a 'fuzzy' fibrillar coat of surface proteins which remarkably appear to be shed during RBC invasion (Cowman, *et al.,* 2012). Since these initial observations, the composition and function of merozoite surface proteins (MSPs) has been of great interest especially in the area of vaccine development as a potential malaria vaccine candidate because of their role in RBC invasion and, more recently, as drug targets for inhibiting blood-stage replication (Boyle, *et al.,* 2013; Chandramohanadas*et al*., 2014).

Merozoite invasion is instigated with an initial interaction of this parasite cell with the erythrocyte surface, followed by a re-orientation step that allows the apical end to interact

with the membrane of the host cell. The contents of the apical organelles, the rhoptries and micronemes, are expelled and a tight junction is formed between the merozoite surface and the erythrocyte membrane(Cowman *et al*., 2002). The tight junction moves along the surface of the merozoite, through a force generated by anactin–myosin motor, until the membrane fuses at the posterior end of the parasite. This results in the formation of a parasitophorous vacuole containing the newly invaded merozoite (Cowman *et al*., 2002).

The initial interaction of the merozoite surface with the erythrocyte membrane must involve proteins on the surface of the parasite and a number of proteins have been characterized. One of these proteins, MSP-1 has been postulated to be involved in the initial interaction of the merozoite with the erythrocytes surface (Holder *et al*., 1992)

Merozoite surface protein 1 (MSP1) has been identified as a target antigen for protective immune responses against asexual blood stage malaria, but effective vaccines based on MSP1 have not been developed so far. The C-terminal proteolytic processing product of merozoite surface protein 1 (MSP1) appears essential for successful erythrocyte invasion by the malarial parasite, *Plasmodium.* The structure comprises two EGF-like domains, and sequence comparisons strongly suggest that the same conformation is present in all species of *Plasmodium*, including *P. falciparum* and *P. vivax*, which are pathogenic in man. The sequence of MSP1 is highly conserved within species (Holder *et al*., 1992).

Other proteins are extrinsically associated with the merozoite surface and are initially located in the parasitophorous vacuole of the schizont stage. These proteins can interact with the merozoites before erythrocyte rupture. These proteins include MSP-2, MSP-3, MSP-6, acid and basic repeat antigen (ABRA), S-Ag and SERA (Cowman *et al*., 2002).

The merozoite has surface coat on its surface which consists of fibrils that are closely spaced (Bannister *et al*., 1986). Each cluster consists of two regions, the outer 40 nM fibril that is 2–3 nM wide and the inner 18–22 nM fibrils, and between the fibrillar coats around is an amorphous proteinaceous covering. Coat filaments occur on the surface of the parasite in regular rows at an early schizont stage, and persist until well after merozoite release. They are sensitive to trypsin and papain, and bind ethanolicphosphotungstate, indicating a proteinaceous nature. According to Bannister *et al*. (1986), they are removed by exposure to phosphate-buffered saline. He also suggested that these filaments correspond to a major merozoite surface protein, and are important in the initial capture of red cells.

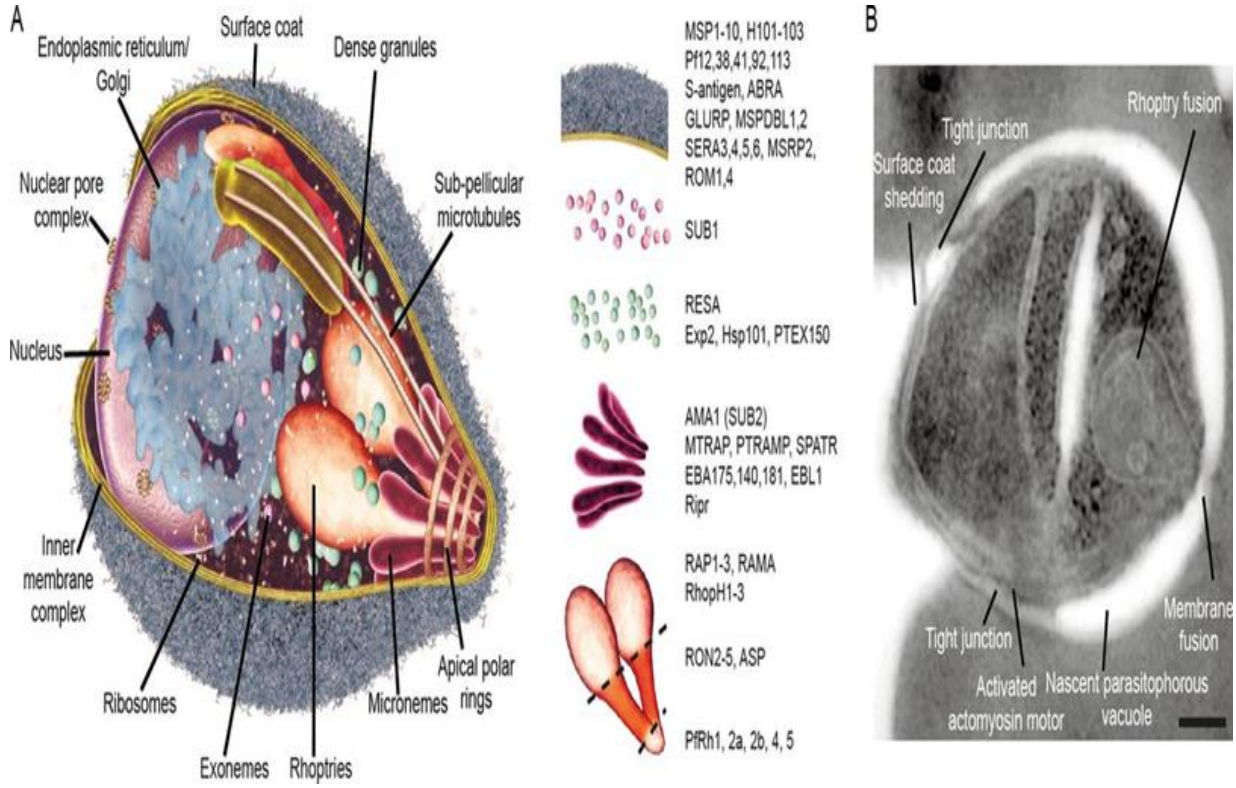



**Figure 3:** Three-dimensional diagram of a merozoite and its core secretory organelles(Cowman*et al.*, 2012)

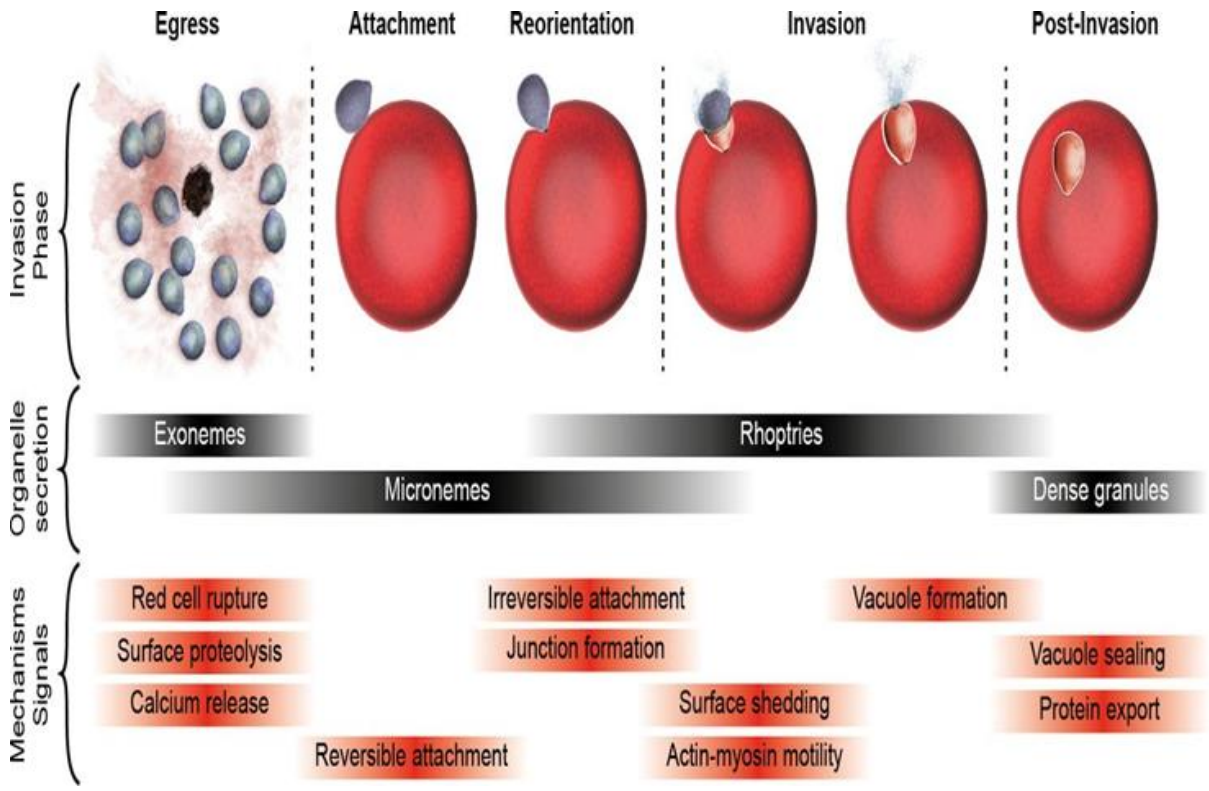



Figure 4. A time course of merozoite invasion of the erythrocyte from egress through post-invasion(Cowman *et al*., 2012)

Merozoite antigens are targets of protective immunity (Ballou *et al*., 2004) and of the ability of antibodies targeting these proteins to block erythrocyte invasion forms has been the rationale behind efforts to generate an effective blood stage vaccine(Cowman *et al*., 2012).

#### 2.5.3.5 Pfs25

Pfs25 is a leading candidate and currently in phase I clinical trial (Chaturvedi*et al*., 2016) Pfs25 has been considered as one of the most vital transmission blocking vaccine candidate antigens, expressed on the surface of zygote and ookinetes. Pfs25 protein is expressed only in the mosquito host and antibody raised against the recombinant Pfs25 protein halts the parasite development within the mosquito vector (Stowers*et al*., 2000). All P25 proteins contain a signal sequence followed by four epidermal growth factor (EGF)-like domains and a C terminal glycosylphosphatidylinositol (GPI) anchor (Lee *et al.*, 2016).

A proposition from gene knockout experiments states that this protein is important for the parasite to survive inside the mosquito midgut. A double-knockout study on *P. berghei* indicates that the loss of P25 antigen reduces the ookinete invasion into the midgut epithelial cells (Chaturvedi*et al*., 2016). An immunogenic form of Pfs25 that was expressed in yeast showed effective transmission blocking activity in membrane feeding assays (Kaslow*et al.*, 1994). Also Intramuscular administration of Pfs25 in mice elicited the potential transmission-blocking antibodies that resulted in more than 90 per cent reduction in oocyst numbers in the mosquito midgut (Lobo *et al*., 1999). ). Trials with clinical grade formulations of Pfs25 molecules involves the preparation of different constructs in immunogenic forms and the testing of safe adjuvant (Epstein *et al*., 2007). Recombinant Pfs25H in conjugation with exoprotein A (EPA) of *Pseudomonas aeruginosa* has been produced and used to make a cGMP pilot lot to use in Phase I human clinical trials in the United States (Shimp*et al*., 2013). A clinical trial of Pfs25-CP VLPs with adjuvant Alhydrogel preparation is ongoing in the United States (https://clinicaltrials.gov/ct2/show/ NCT02013687) ((Chaturvedi*et al*., 2016), this is based on the report of Jones et al. 2013 which showed that administration of one or two doses of Pfs25-CP VLPs with adjuvant Alhydrogel® in mice stimulates the antibodies that have shown absolute transmission blocking activity (Jones *et al*., 2013).

### 2.5.3.6 Glutamate- Rich Protein (Glurp)

Glutamate-rich protein is a *Plasmodiumfalciparum* (Pf) antigen found in all stages of the parasite and has been reported to induce clinical immunity (Duru and Thomas, 2014). GLURP antigen is one of the recent approaches towards the development of multi-stage malaria vaccine which can target both transmission and asexual parasite life cycle stages(Chaturvedi*et al*., 2016). The *P. falciparum* glutamate-rich protein (GLURP) is a vaccine candidate that has been evaluated in Phase I trials. Immunoepidemiological studies performed in both high and low malaria transmission areas have revealed a great prevalence of GLURP antibodies in adults (Pratt-Riccio*et al*., 2005), as well as a substantial association between high GLURP-specific antibody levels and low parasite densities (Pratt-Riccio*et al*., 2013). Furthermore, a contemporary study of controlled experimental *P. falciparum* infections demonstrated that non-immune individuals acquire GLURP antibodies following a single, brief low-density *P. falciparum* infection (Turner *et al*., 2011). GLURP is located on the extracellular merozoite surface and are involved in red blood cell invasion, although it is expressed in both the pre-erythrocytic and erythrocytic stage (Turner *et al*., 2011). The gene is located on chromosome 10 and it encodes a polypeptide of 1271 residues with a proposed molecular mass of 145 kDa (Borre*et al*., 1991). The gene encoding GLURP in *P. falciparum* consists of three regions namely, N-terminal non-repetitive region (R0), central repetitive region (R1) and an immunodominant C-terminal repetitive region (R2) (Kumar *et al*., 2014).

A recent study on naive Dutch volunteers by Turner *et al*., showed that antibody level against R2 repeat region of GLURP in plasma was found much higher than that of R1 and R0 regions (Turner *et al*., 2011). The studies additionally suggested that R2 repeat region plays an important role in inducing protective immunity against *P. falciparum* malaria. The R2 repeat region has been reported as a good genetic marker for *P. falciparum* genotyping and also for differentiating new infection from recurring infections (Barrer*et al*., 2010).

Studies performed in areas highly endemic for malaria have demonstrated a high prevalence of antibodies against two well-defined regions within *P. falciparum* GLURP, the relatively conserved N-terminal non-repeat region (R0) and the immunodominant repeat region (R2). Predictions of the most hydrophilic and antigenic domains of GLURP identified some potential B cell epitopes in regions R0 (P1, P3, P4, P5, P8, P9, P10, P11, and S3) and R2 (S4). (Pratt-Riccio*et al*., 2005). GLURP play a primary role in protection against *P. falciparum* malaria by effector mechanisms such as antibody-dependent cellular inhibition (ADCI), and the collaboration of monocytes and antibodies impairing parasite multiplication (Oeuvray*et al*., 1994). B-cell epitopes identified in a study by Theisen*et al*., demonstrated that antibodies to all of these epitopes were found able to promote a monocyte-dependent inhibition of parasite growth (Theisen*et al*., 1998). Observations, in various field studies propose that glurp R0 region is conserved while the R2 region is heterogeneous. This conclusion, however, have been supported by *in-vitro* assays showing that the *Pf* glurp gene displays some antigenic diversity, with differing allelic variants (Duru and Thomas, 2014)

Like every other potential malaria vaccine candidate, efforts to develop a malaria vaccine using GLURP antigen have been hampered due to extensive antigenic diversity of the protein, with the degree of diversity directly related to geography and transmission intensities.

Purified recombinant circumsporozoite protein vaccine, RTS, S/AS01

The research on the RTS,S vaccine started in 1984 by scientists working at GlaxoSmithKline's (GSK) laboratories, resulting in the most advanced malaria vaccine to date (MVIand GSK, 2015). RTS,S was identified as a potential candidate for further development following encouraging results in an experimental challenge study and successive phase 2 studies in adults and children which showed that the vaccine was safe,

was immunogenic, and provided protection against clinical episodes of malaria in the range of 30%–60%.(The RTS,S Clinical Trials Partnership, 2014)

### 2.6 Antimalarial Drug Resistance

Drug resistance by *P. falciparum* is the ability of a parasite strain to survive or multiply despite the administration and absorption of a drug when given in doses equal to or higher than the standard minimum inhibitory concentration (MIC)

Antimalarial drug resistance is a major public health problem which hinders the control of malaria. Several factors influence the emergence and spread of drug-resistant parasites: the intrinsic frequency with which the genetic changes occur, the degree of resistance conferred by the genetic change, the proportion of all transmissible infectious agents exposed to the drug (selection pressure), the number of parasites exposed to the drug, the concentrations of drug to which the parasites are exposed, the pharmacokinetics and pharmacodynamics of the antimalarial medicine, individual (dosing, duration, adherence) and community (quality, availability, distribution) patterns of drug use, the transmission intensity (White and Pongtavornpinyo, 2003). The measurement of drug resistance in malaria is complex. Four different tools can be used:

1. Therapeutic drug efficacy studies measure clinical and parasitological efficacy and are the primary source to inform the treatment policy of the national malaria control programme (NMCP)

2. *In-vitro* studies measure the intrinsic sensitivity of parasites to antimalarial drugs

3. Molecular marker studies identify genetic mutations and subsequently confirm the presence of mutations in blood parasites

4. Pharmacokinetic studies characterize drug absorption and drug action in the body (WHO, 2010).

Resistance to antimalarial medicines is a recurring problem. Resistance of *P. falciparum* to previous generations of medicines, such as chloroquine and sulfadoxine-pyrimethamine (SP), became widespread in the 1970s and 1980s, undermining malaria control efforts and reversing gains in child survival.

The first drug used to treat malarial fever in the western hemisphere was quinine, which has been known for more than 350 years (Kremsner and Krishna, 2004). It has been the only antimalarial medication until the beginning of the 20th century. Chloroquine was developed in 1934 and has been used worldwide on a large scale as first-line treatment against malaria infections. In 1957, the first cases of resistance were observed on the Thai-Cambodian border and in the following years also in South America and Africa. In the next decades, nearly all countries have to change their recommendations of first line treatment for uncomplicated *falciparum* malaria to sulfadoxine-pyrimethamine (SP).

### 2.6.1 Malaria drug markers

#### 2.6.1.1 *Plasmodiumfalciparum*chloroquine resistance transporter (*Pfcrt*)

*Pfcrt* (*Plasmodiumfalciparum*chloroquine resistance transporter) is a protein that is found on the surface of the digestive vacuole (DV) membrane. It is aputative transporter or channel that belongsto DMT family of transporter proteins (drug metabolitetransporter). Over the years it has been reported to be an essential *tool in conferring chloroquine resistance to Plasmodiumfalciparum(Ibraheem et al., 2014).* The role of *pfcrt* in the efflux of CQ outside

the DV has been extensively studied in lots of *in-vitro* cross-genetic transfection research. The outcomes ended up with controversial conclusions stating that CQ resistance is dependent on *pfcrt* expression level while some other studies failed to prove it (Waller *et al*., 2003; Zhang *et al*., 2002). The controversy on whether CQ resistance is dependent on *pfcrt* gene expression level was resolved by the results from the work of Sidhu and colleagues who reported that CQR is more related to point mutations in *pfcrt* gene rather than *pfcrt* expression level (Sidhu *et al.*, 2002).

*Pfcrt* gene is a protein with molecular weight of 48.6 kDa which consists of 424 amino acids arranged in 10 $\alpha$-helical transmembrane domains (TDMs) oriented inside the DV membrane and N-termini which are exposed to the cytosol (Ibraheem *et al*., 2014). The 424 amino acids are encoded by a 13 exon gene in the chromosome having 36-kb segment (Fidock *et al.*, 2000). Inside the structure of *pfcrt* protein, there are 32 candidate codons that can undergo point mutations that may confer for changes to *pf*crt function (Ibraheem *et al*., 2014).

*Pf*crt function as a transporter protein is to facilitate efflux of alkaloids, amine compounds, divalent cations and amino acids as well as peptides that result from the vacuolar digestion of globin. It is also involved in the regulation of H+ homeostasis (Sidhu *et al.*, 2002).

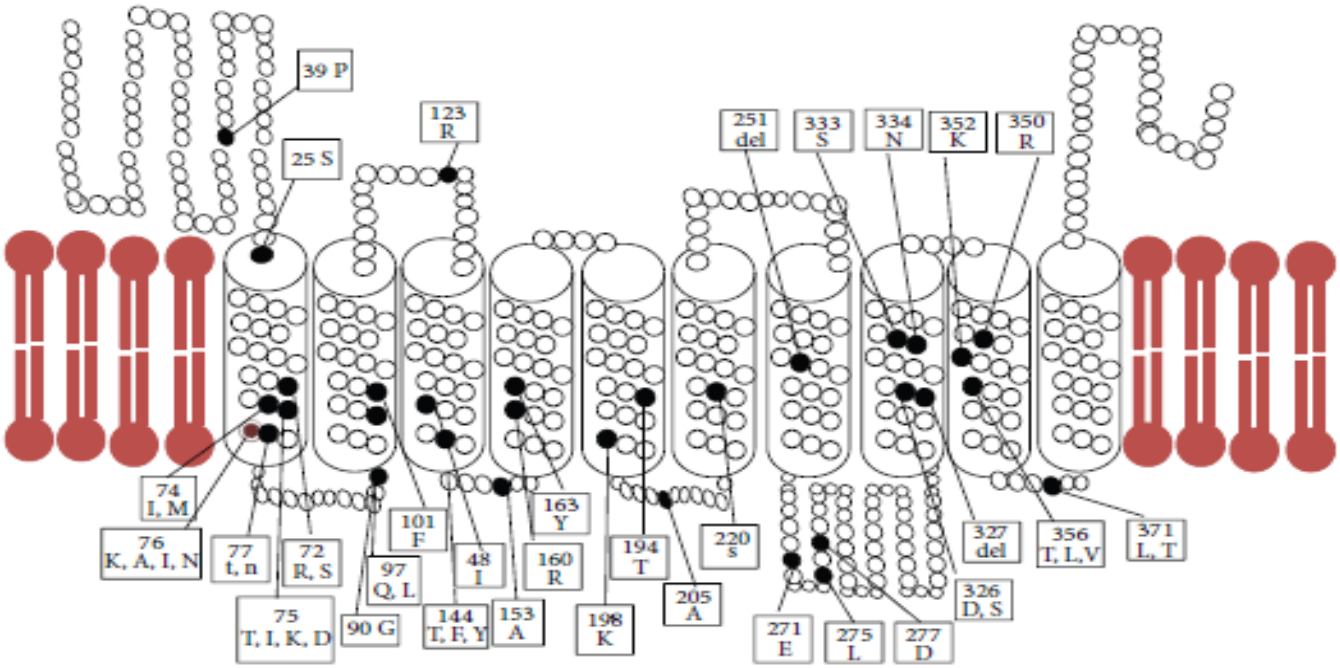



Figure 5. Structure of *Pfcrt* protein showing codons with point mutation

Source: (Ibraheem *et al*., 2014)

**2.6.1.1.1 *Pfcrt* Mutations and Drug Resistance:**

Point mutations that alters the amino acid sequence of any channel protein results in changing of its physiochemical properties and functional characters. This is because each amino acid is responsible for certain distinguishing properties such as molar mass, van der Waals volume and average volume of buried residue, lipophilicity or hydrophobicity index, and isoelectric point which determines its effect on the channel function (Ibraheem *et al*., 2014). These properties according to Ibraheem *et al*. affect channel function through changing its side chain volume, negativity, and polarity. The majority of the mutations take place at the side that faces the cytosol. Ordinarily, DV pH is sustained at narrow range, which is more or less around 5. When IP value approaches or drops below 5 due to the presence of amino acids

whose isoelectric point is below 5, higher channel negativity is conferred and thus, the incidence CQ evacuation outside the channel (Antosiewicz *et al.,* 1994). Another suggested mechanism of action is based on the hypothesis of Bray *et al.,* 2005, that PfCRT facilitates the diffusion of the charged drug species which is also known as "charged drug leak" hypothesis. Within the acidic DV, the drug molecules present are charged as compared to the outside of the DV, which neglects the drug binding. The charged molecules require some kind of carrier, as they cannot pass through the hydrophobic environment of a membrane. This explains the significance of the K76T mutation in which the lysine in wild-type CQS isoforms has a basic side group having positive charge that repels protonated CQ, while the neutral threonine allows for an open pore through which charged CQ may pass.(Bray *et al*., 2005).

The polymorphisms of the PfCRT gene produces two different forms of *Pfcrt* with respect to the type of response given to drug such as Chloroquine Sensitive (CQS) and Chloroquine resistance (CQR). According to Parida *et al.* the point mutations of the *Pfcrt* involved in CQR include codons, 72–76, 271, 326, 356, and 37 (Parida *et al.,* 2016). Different mutations in the PfCRT gene which change the nucleotide sequence into different genes and hence different haplotypes, which are very remarkable in the incidence of chloroquine resistant.

Since the detection of *Plasmodiumfalciparum*chloroquine (CQ) resistance transporter (PfCRT) and its role as the primary genetic determinant of chloroquine resistance (CQR) in *P. falciparum*, 53 distinct isoforms of this 424 amino acid protein have been found to be expressed in parasite isolates from around the world which is posited to have descended from at least five independent 'founder events' in four regions: Southeast Asia (SEA), South America (SA) (two events), Papua New Guinea (PNG), and the Philippines probably due to variable drug selection pressure in these regions (Callaghan *et al.,* 2016).

Although CQ has been withdrawn as an approved treatment against *P. falciparum*malaria,

mutant CQR-conferring PfCRT isoforms have persisted in some regions (especially within SA)(Sá *et al.*, 2009).

Recent works showed that some mutant PfCRT isoforms are revertants that do not confer CQR at all (Callaghan *et al.*, 2015; Goswami *et al*., 2014; Koleala *et al.*, 2015). This needs to be validated to provide inference tools with respect to current drug use policies and might be useful in the interpretation of data from a variety of earlier field based studies.

*According to* Callaghan *et al.,* 2016, Presence of the K76T mutation within PfCRT has until very recently been considered 'diagnostic' for a CQR phenotype hence the CQR vs CQS status of parasite isolates has often been assigned based on the presence of the key PfCRT K76T mutation alone. Callaghan and colleagues also suggested that it is crucial to monitor the continued evolution of PfCRT protein both to inform an understanding of its interaction with currently used anti-malarials and to assess potential re-introduction of CQ as a regional treatment (perhaps as a companion drug in geographically constrained combination therapy).

#### 2.6.1.2 *Plasmodiumfalciparum* Multidrug Resistance gene 1*(Pfmdr1)*

*Pf*mdr-1 is a protein that is abundant on digestive vacuole membrane of *Plasmodiumfalciparum,* which is involved in multidrug resistance via sending out of xenobiotics away of the cytosol *(Ibraheem et al., 2014). It belong to the group of transporters known as* P-Glycoprotein Transporters orABC transporters (ATP dependent cassette transporters). In *Plasmodium,* it acts as an secondary mechanism through which drug gain entrance into the DV beside simple. *Pfmdr1*, which encodes a transmembrane glycoprotein (Pgh1, for P-glycoprotein homologue 1), and the multidrug-resistant (MDR) phenotype was first reported in 1989 (Price *et al*., 1999). Point mutations in *pfmdr1*, most notably at codon 86, have been implicated in decreased chloroquine sensitivity (Viana *et al.,*

2006). According to Viana *et al.* Polymorphism in the Asn86Tyr codon has been related to chloroquine resistance in *P. falciparum*isolates in Nigeria, Guinea-Bissau,Gambia, and Malaysia.

*P. falciparum* has two mdr-like genes (pfmdr 1 and pfmdr 2) located at chromosomes 5 and 14 respectively (Viana *et al.,* 2006).*Pfmdr1* has been linked to the chloroquine resistance phenotype while *Pfmdr 2* has been linked to translocation of heavy metals and has nothing to do with multidrug resistance *(Ibraheem et al., 2014).Pfmdr1* encodes a protein of 160,000 Daltons that is expressed at higher levels in a chloroquine resistant cloned isolate. The *Pfmdr 2* gene is located on chromosome 14 and it is in equal copy number in chloroquine resistant and sensitive isolates.

**2.6.1.2.1 *Pfmdr1* and drug resistance.**

Chloroquine (CQ) resistance has been associated with mutations in the *Pfmdr1* gene, particularly at codons 86, 1042 and 124(Gamo, 2014).*Pf*mdr-1 point mutations have been observed in both CQ resistant and susceptible strains of *Plasmodiumfalciparum.* They ablate the transporter capacity to drift drugs, namely, CQ, quinoline (QN), mefloquine (MQ), halofantrine (HF), and lumefantrine (LM), into the DV (Ibraheem *et al*., 2014). Four likely single nucleotide polymorphisms (SNPs) has been identified in *Pf*mdr-1 gene as reported by Tinto*et al*. 2008. They include N86Y, N1042D, S1034C, and D1246Y in which asparagine at codons 86 and 1042, serine at codon 1034, and aspartic acid at codon 1246 of *pf* mdr-1 protein had been replaced by tyrosine, aspartic acid, cystine, and tyrosine, respectively.

The work of Mvumbi and his colleagues in 2015, showed that among five point mutations that were explored on codons positions 86, 184, 1034, 1042 and 1046 that N86Y mutation was present in 66.7 % of samples studied (Mvumbi *et al*., 2015).

Price *et al.,* 2004 showed that mutations in and, more importantly, amplification of the wild-

type *pfmdr1* gene confer resistance to the 4-methanolquinoline mefloquine, presumably through an increased ability to efflux the drug out of the DV (Price *et al*., 2004)

(Duraisingh and Cowman, 2005) suggested that *Pfmdr1* geneconfer resistance to antimalarial drug with respect to its localization within the parasite vacuole from where it may regulate intracellular drug concentrations.

*Pfmdr1* mutations are said to assist the chloroquine-resistant parasites by augmenting the level of resistance as reported by Nneji and his colleagues. Mutations in the Pfcrt gene appear to be necessary for chloroquine resistance in-vitro and for chloroquine treatment failure *in-vivo*. Other factors such as host immunity and other parasite genes, including *Pfmdr1* are important in determining clinical outcome (Nneji *et al.*, 2015).

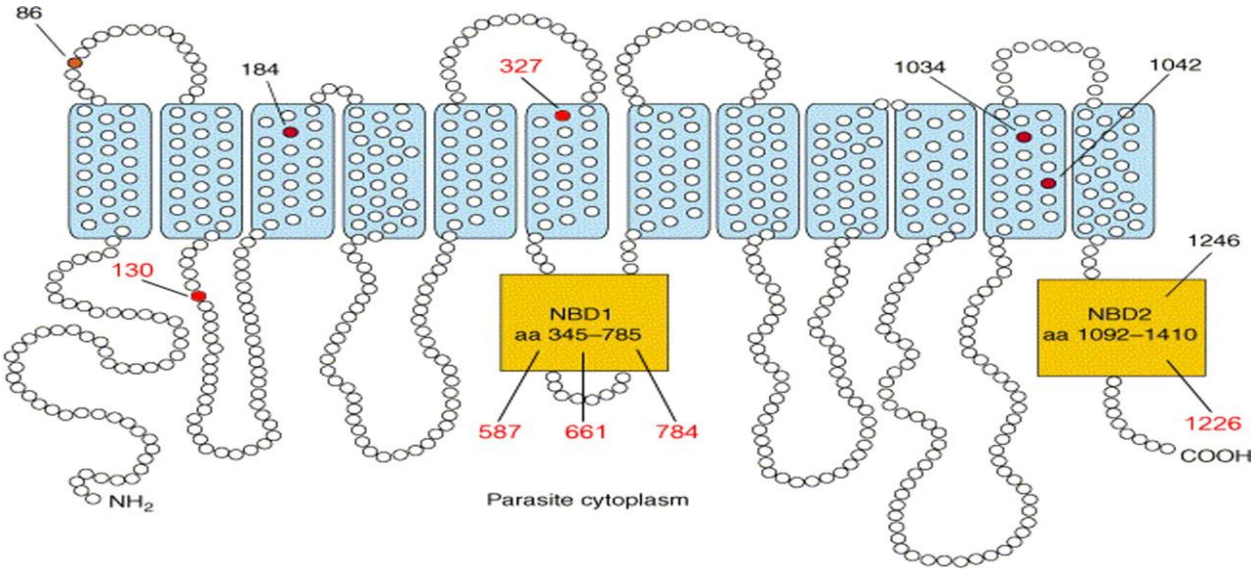



Figure 6: Predicted structure and representative haplotypes of *P. falciparum* multidrug resistance transporter.

Source:(Imwong *et al*., 2010)

### 2.6.1.3*Plasmodiumfalciparum* dihydrofolate reductase(*Pfdhfr*)

*Pfdhfr* is a protein encoded by the *Pfdhfr-ts* gene, which has one exon located on chromosome 4. The gene has molecular mass of 71.73 kDa and 608 amino acids in length. *Pfdhfr* is a bifunctional enzyme involved in two main folate metabolic activity: the biosynthesis of dTMP by thymidylate synthase activity and the reduction of dihydrofolate into tetrahydrofolate by dihydrofolate reductase activity (Antony and Parija, 2016). The folate mechanism of PfDHFR enzyme in the biosynthesis of nucleic acid is inhibited by the action of antifolate drugs such as pyrimethamine and cycloguanil, thus reducing the production of pyrimidine for DNA replication (Hyde, 2005). So many works as reported by Antony and Parija, 2016 has linked Pyrimethamine resistance to point mutation in the PfDHFR protein at S108D codon, further mutation at N51I, C59N, and I164L as well as amplification of gene.

Based on amino acid sequence alignment and crystal structures of *Pfdhfr-ts* Yuvaniyama *et al*., 2003, showed that *Pfdhfr* domain contain a unique N-terminal protrusion of six amino acids and two additional inserts with respect to DHFR of human and other organisms, designated Insert-I (residues 20–36) and Insert-II (residues 64–99)

In the crystal structures of *Pfdhfr-ts*, the N-terminal region forms a short α-helix that interacts with the surface of the DHFR domain and anchors Insert-II helix to the protein core structure, while Insert-I is involved in inter-domain interaction with the TS unit(Japrung *et al.*, 2005).

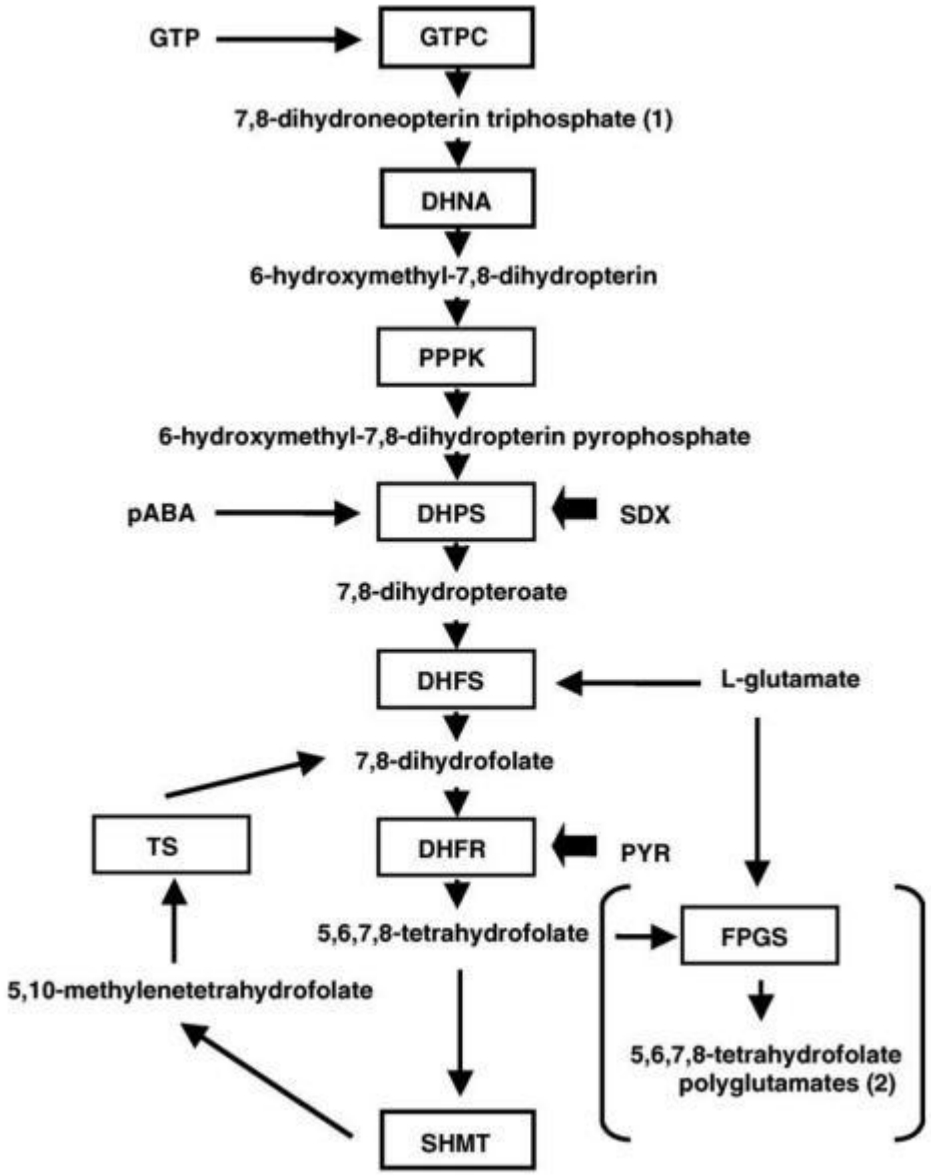



Figure 7.Principal enzymes and substrates of the folate pathway involved in formation of tetrahydrofolate (THF) and its utilization in the thymidylate cycle

Source: (Hyde, 2005).

**2.6.1.3.1 Pfdhfr and Drug resistance:**

Resistance of *Plasmodiumfalciparum* to antifolates is an important problem in antimalarial

chemotherapy and has been shown to be associated with mutations in the Dihydrofolate reductase (DHFR) domain.

Double mutation at A16V and S108T positions in PfDHFR linked with the resistance of *P. falciparum* to cycloguanil (Hyde, 2007).

Mutation to Asn108 leads to resistance to Pyrimethamine and a moderate loss of response to cycloguanil (CYC). Also a mutation of Threonine 108 along with Ala16 to Val 16 mutation provides resistance to CY C (Rao and Tapale, 2013). These findings according to Rao and Tapale, 2013, strongly suggest that the threonine-108 side chain in the *P. falciparum* enzyme makes an important interaction with the chlorophenyl group of pyrimethamine. Disruption of this interaction hence leads to drug resistance.

Consequently, the high frequencies of resistant parasite populations have been attributed to increased Pyrimethamine consumption. An Asp3Ile substitution at codon pfdhfr51 (pfdhfrN51I) and/or a Cys3Arg exchange at codon pfdhfr59 (pfdhfrC59R) appears to enhance Pyrimethamine resistance if one or both of these occur concurrently with Pfdhfr S108N. *Pfdhfr*S108N-N51I-C59R is the combination of mutations most strongly associated with Pyrimethamine resistance (Marks *et al*., 2005)

#### *2.6.1.4 Plasmodiumfalciparum*dihydropteroate synthase(*Pfdhps)*

*Pfdhps* is a gene located on chromosome 8 with three exons that encode for *Pfdhps* protein. It consists of 706 amino acid and 83.37 kDa molecular weight (Antony and Parija, 2016). PfDHPS enzyme catalyzes the reaction of the p-aminobenzoic acid (PABA) with a pterin derivative in synthesizing dihydrofolate, a folate precursor that is essential for the synthesis of pyrimidine in the parasite (Foote and Cowman, 1994). This catalytic enzyme action to synthesize dihydrofolate is inhibited by sulfa drugs (sulfadoxine and dapsone), which act as an analog to PABA.

The DHPS gene cloned from *P. falciparum* has been shown to encode a putative bifunctional enzyme with 7, 8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK), the enzyme before DHPS in the folate pathway. Rattanachuen *et al*., 2009, reported the case of a De novo folate biosynthesis in the malaria parasite which occurs through sequential reactions catalyzed by a number of enzymes which involves DHPS. However, they suggested that the bifunctionality that characterized the new enzymes might facilitate substrate channeling within the same subcellular compartment localization or help to regulate the protein functions, and inter- or intra-domain communication.

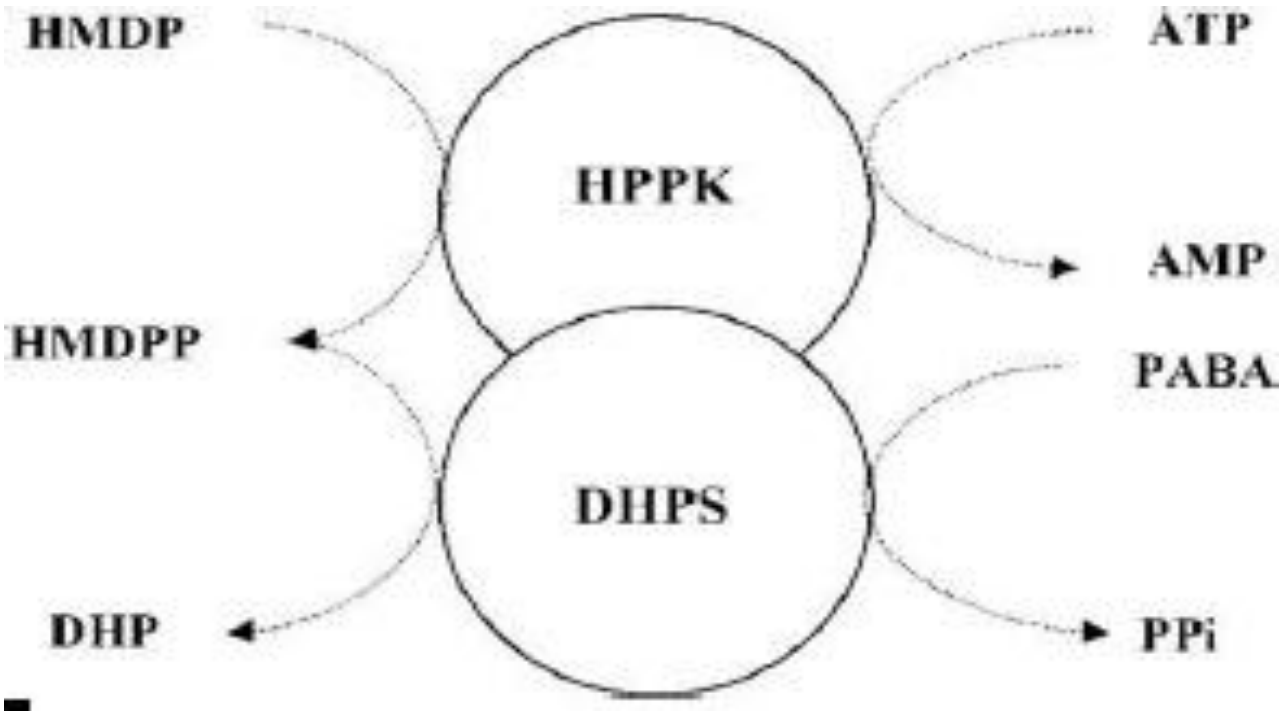



Figure 8 : Reactions catalyzed by HPPK and DHPS of ***Plasmodiumfalciparum***

Source: (Rattanachuen *et al*., 2009)

#### 2.6.1.4.1*Pfdhps* and drug resistance

Biosynthesis of folate is inhibited by two groups of compounds; sulfonamides and antifolates.

Sulfonamides are competitiveinhibitors of DHPS, whereas antifolates target the enzyme dihydro-folate reductase (DHFR). Because of their synergistic action, both sulfonamides and antifolates have been used in combination in the treatment of malaria. Resistance occurs mainly by mutations of genes coding for the target enzymes that cause a reduction in drug binding.

Five mutations in the PfDHPS protein (S436A/F, A437G, L540E, A581G, and A613T/S) have linked with resistance to sulfadoxine in *P. falciparum*(Antony and Parija, 2016) Mutation at 436, 581, and 613 codons are associated with higher level of resistance, whereas mutation at 437 and 540 contribute a low level of resistance with modulation effects in association with other mutation in PfDHPS. Since the antimalarial drug resistance as monotherapy has emerged, sulfadoxine is always provided in combination with pyrimethamine, known as SP or Fansidar, and resistance to SP was associated with point mutation in both *Pfdhfr* and *Pfdhps* gene (Petersen *et al.*, 2011).

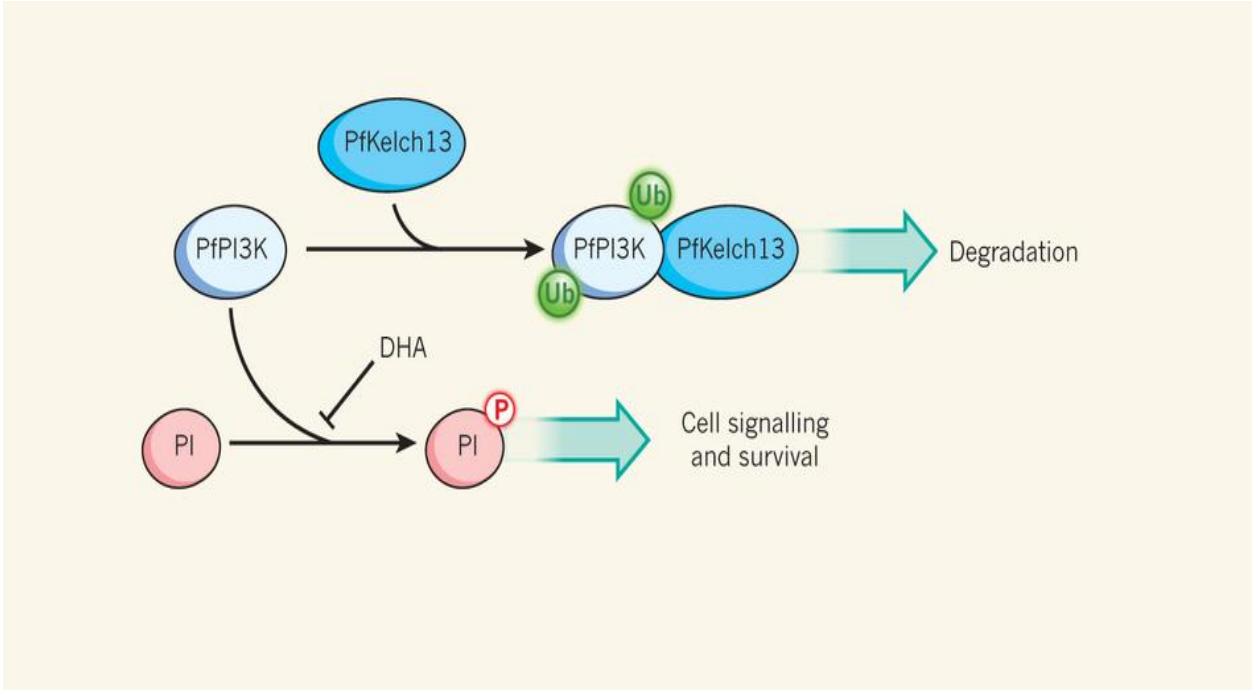
### *2.6.1.5 Plasmodiumfalciparum Kelch13 (Pfkelch13)*

*Plasmodiumfalciparum* gene kelch13 (**Pfkelch13**) codes for K13 protein which has one exon located on the chromosome 13 with 726 amino acids and 83.66 kDa molecular mass. The protein has a C-terminal region with six kelch motifs consisting of beta sheets that folds into a propeller domain and mutation in this region is predicted to disrupt the domain scaffold and alter its function (Antony and Parija, 2016). *PfKelch13* protein regulates the level of PfP13K. It functions by binding to PfPI3K and mediates the addition of ubiquitin groups (Ub), tagging

PfPI3K for degradation. PfPI3K is required to phosphorylate (P) the phospholipid phosphatidylinositol (PI) to form phosphatidylinositol 3-phosphate, promoting cell signaling and survival (Mbengue *et al*., 2015). Mbengue and his colleagues claimed that dihydroartemisinin (DHA), the active ingredient of artemisinin-based antimalarial drugs, inhibits PfPI3K activity in ring-stage parasites (Mbengue *et al*., 2015).

Consequently, mutations in *PfKelch*13 prevents dihydroartemisinin (DHA), the active ingredient of artemisinin-based antimalarial drugs, from binding to PfPI3K thus increasing PfPI3K levels and so help parasites to overcome the effects of DHA.

Even though Mbengue and his co-workers tried to provide the missing information on how *PfKelch*13 is associated with DHA resistance, Burrows, 2015, noted that their work provided no evidence that the malaria parasite separates itself from DHA by destroying it or expelling it from its site of action, nor that the parasite hides in a protective state until DHA has




dissipated (Burrows, 2015).

*Figure9: PfKelch13 Function and* Resistance Mechanism.

Source: (Mbengue *et al*., 2015).

### *2.6.1.5.1 PfKelch13 and Drug Resistance:*

Drug resistance can arise in various ways namely;

- a biological target can change or mutate so as to reduce a drug's ability to bind to it;
- the drug can become physically separated from the target, such that it can no longer exert its effect;
- or the biological target can increase so high enough levels to overcome the presence of the drug.

Mbengue and colleagues show that dihydroartemisinin (DHA) is a potent inhibitor of a *P. falciparum*enzyme called phosphatidylinositol 3-kinase (PfPI3K), which phosphorylates an important phospholipid, phosphatidylinositol (PI), to produce phosphatidylinositol 3-phosphate (PI3P) in ring-stage parasites.

The mechanism of action suggested by Mbengue *et al*., 2015 is specific to ring-stage parasites, implying that other reported biological consequences of DHA that affects the parasite at other stages of its life cycle has not been properly accounted for.

Burrows, 2015 agreed that*PfKelch*13 mutations correlate with only a slight increase in levels of PfPI3K in resistant parasites, but the extent of ring-stage resistance to DHA seems to be of a high magnitude, indicating that small alterations in PfPI3K levels can have a large effect on resistance. Thus implying that resistance to DHA could be overcome by increasing the dose of artemisinin in drug combinations, to inhibit PfPI3K more potently and such a study has

been independently conducted by Das *et al.,* 2013. The conclusion was that increasing the artesunate treatment dose up to 8 mg/kg/d or splitting the dose does not improve parasite clearance in either artemisinin resistant or more sensitive infections with *P. falciparum*(Das *et al*., 2013)

Artemisinin resistant *P.falciparum*has been detected in five countries in the Greater Mekong sub-region (GMS), and its associated with slow parasite clearance arising from resistance to selected for partner drug resistance (WHO, 2016; White, 2016) . Imwong and colleagues provided evidence of a hard selective sweep of the *PfKelch13* C580Y mutation, which has spread from western Cambodia to northeastern Thailand and southern Laos (Imwong *et al.*, 2017)

Elizabeth suggested that finding a dominant artemisinin resistant haplotype that is associated with partner drug resistance has implications for malaria control and ACT resistance containment strategies and recommended that careful monitoring for the PfKelch13 C580Y haplotype in neighbouring countries, as well as India and west and east Africa should be done to identify further spread of artemisinin resistance should it occur (Hemming-Schroeder and Lo, 2017)

In contrast to the report of Imwong, Balikagala suggested that mutations in *pfkelch13* and the six background genes may not play an important role in the *in-vivo* selection after artemether–lumefantrine treatment in Uganda. He posed that different mechanisms might rather be associated with the existence of parasites after treatment with the said drug (Balikagala *et al*., 2017).

Futhermore, point mutation in the propeller region of K13 protein has been identified as a key determinant for artemisinin resistance in *P. falciparum*. Nonsynonymous polymorphism at Y493H, R539T, I543T, and C580Y position observed in the kelch repeat region of K13

propeller domain have been associated with higher resistance to artemisinin (Ariey *et al*., 2014)

#### 2.6.1.6 *Plasmodiumfalciparum* Adenosine Triphosphatase (*PfATP6)*

**PfATP6**, also known as PfSERCA or PfATPase6, is a calcium ATPase gene encoded by the malaria parasite *Plasmodiumfalciparum* .The protein is thought to be involved in calcium ion transport (Arnou *et al*., 2011). *P. falciparum* genome contains a single SERCA gene,

originally described in 1993 by Kimura *et al.*, 1993. The coding region of the gene consists of 3684 nucleotides and corresponds to a protein with a molecular mass of 139.4 kDa. Judging from the amino acid sequence alignments, Arnou *et al.,* claimed that PfATP6 conserves all of the motifs and residues that are important for the function and/or structure of a SERCA pump such as two high-affinity $Ca^{2+}$ -binding sites, a nucleotide-binding site and a phosphorylation site.

PfATP6 folds with a membrane domain containing ten transmembrane helices and a cytosolic part with well-defined catalytic domains (Laursen *et al.,* 2009).

PfATP6 contains insertions that have high frequency of asparagine residue in addition to two polyasparagine motifs (nine and ten homopolymericruns). The presence of such extraordinary stretches is a characteristic feature of the proteins present in the parasite, which makes it different from any other form of P-type ATPases (Aravind *et al.,* 2003).

Arnou *et al*., 2011, while reporting that the exact functional role of the additional insert in PfATP6 that makes it a distinctive pump from other P-type ATPases remains unknown, he suggested that the inserts could facilitate the immune evasion by the parasite by generating a non-productive antibody response against the asparagine-rich regions of *Plasmodium*proteins

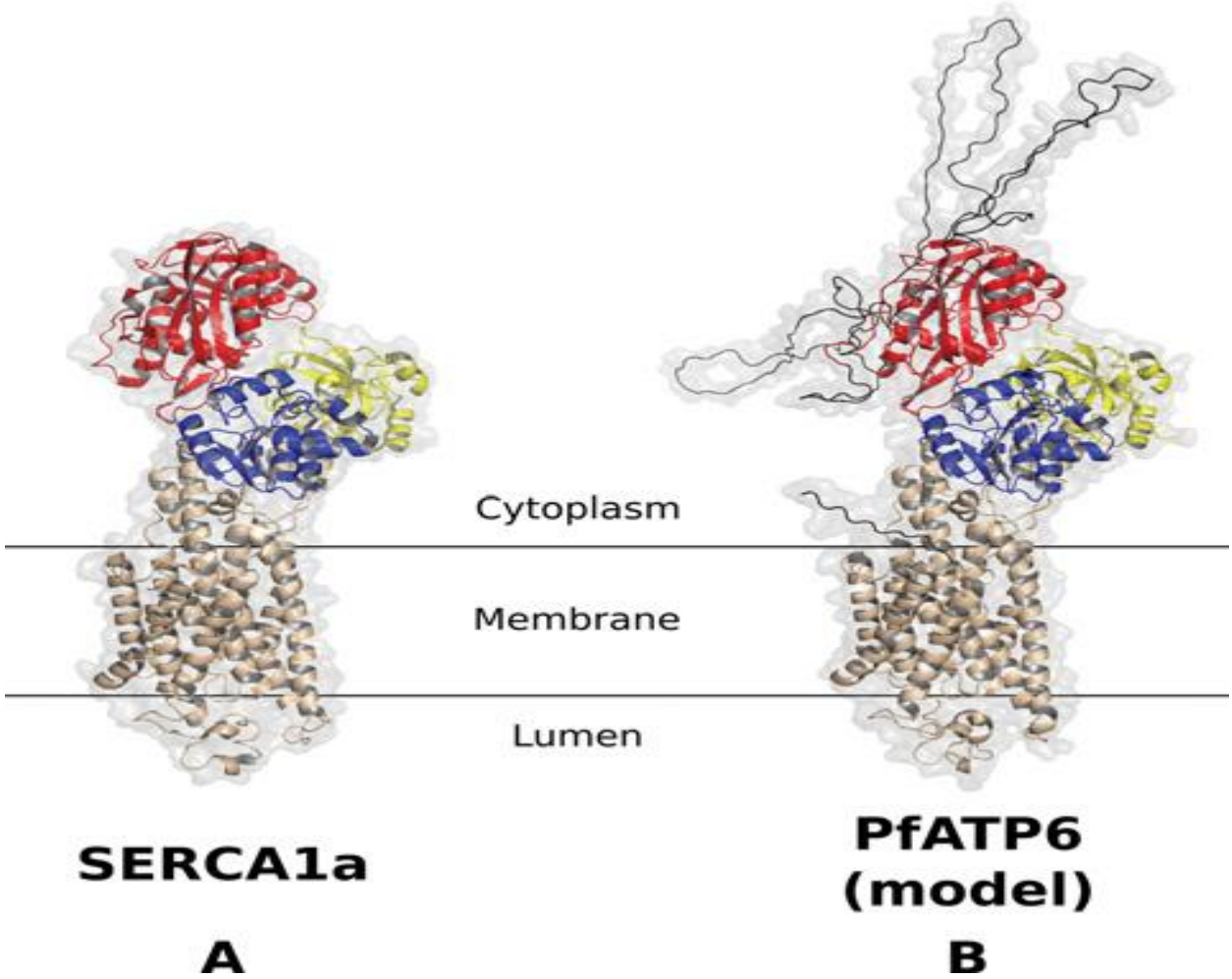



Figure 10 : Comparison of SERCA1a and PfATP6 structures

Source: (Arnou *et al*., 2011).

**2.6.1.6.1 PfATP6 and Drug resistance:**

Krishna and colleagues showed that artemisinin is transported from the red blood cell into the

parasite inside parasite-derived membrane vessicles (Krishna *et al*., 2014). Once inside the parasite, artemisinin is activated by free iron, or another iron-dependent process, that occurs close to *PfATP6* in the endoplasmic reticulum. The activated artemisinin specifically and irreversibly binds and inhibits *PfATP6*, and inhibits parasite growth as shown in the figure below.

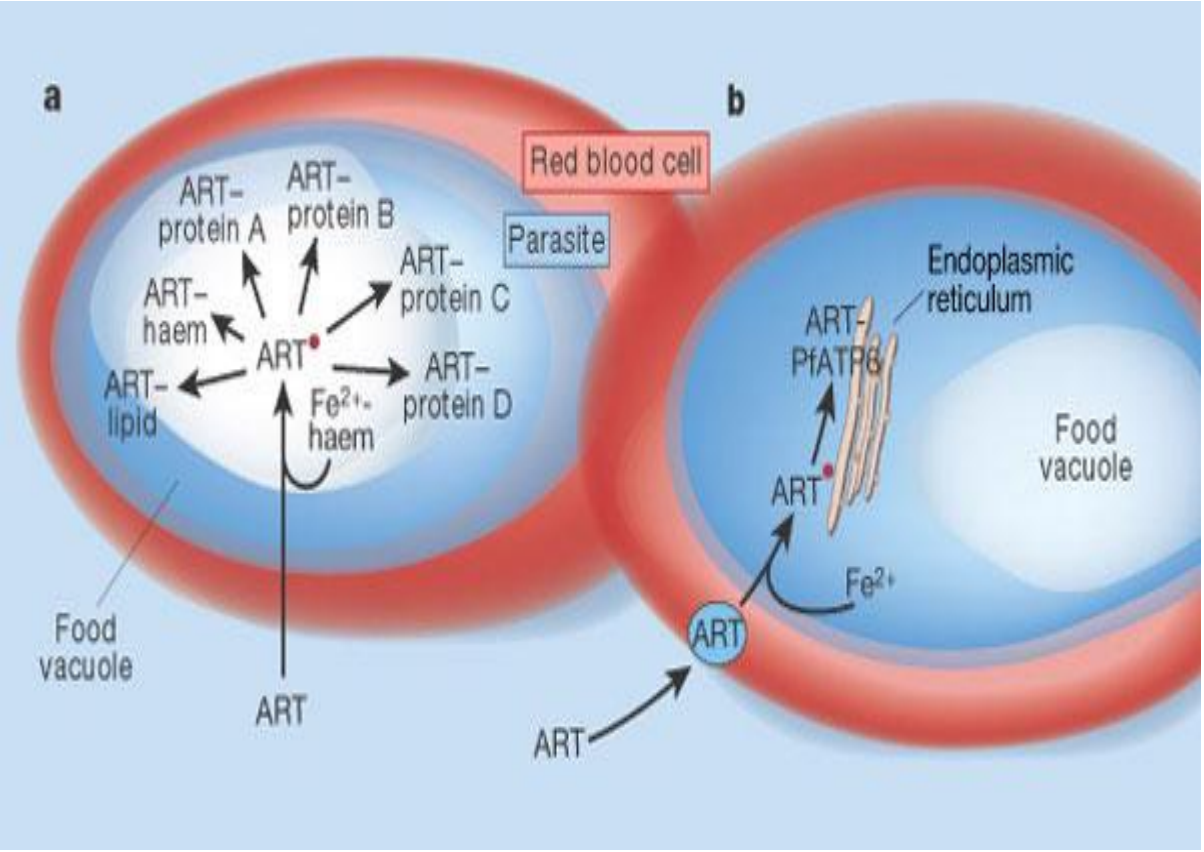



Figure 11: Mechanism of action and Molecular targets of ART

Source (Ridley, 2003)

Artemisinin and its derivatives are most effective antimalarial drugs which act by selectively inhibiting *Plasmodiumfalciparum ATPase6* protein, the only SERCA-type Ca2+ *ATPase* in

the *P. falciparum*genome.(Sharma *et al.,* 2014). Sharma and his colleagues believed that *ATPase6* protein in *P. falciparum*is the primary target for artemisinins. Jambou *et al*., 2005 and several other researchers has established that serine to asparagine mutation at codon 769 in *P. falciparum ATPase6* gene is associated with raised artemether half maximal inhibitory concentration (IC50). Thus making mutations in this protein to be used as a molecular marker for monitoring artemisinin resistance.

Based on heterologous expression studies and biochemical assays, *PfATP6* has been postulated to be a prime target of ART, and L263E was considered a potential mutation that mediates ART resistance (Cui *et al*., 2012). The L263E mutation according to Cui *et al.,* 2012 was proposed based on docking simulation and found to confer insensitivity to ARTs in the *Xenopus* oocyte system.

*Invitro* susceptibility tests have not been carried out on most of the identified SNPs. However, Only four of the forty-four PfATPase6 SNPs according to Afoakwah *et al.,* 2011, have been shown to confer some level of resistance to artemisinins. The L263E mutation has been recorded only by laboratory engineering and not yet in any wild parasite/field isolate (Afoakwah *et al.,* 2011)

Furthermore, S769N, A623E and E431K mutations, have been reported in field isolates and their occurrence is very rare with the exception of the E431K mutant, which has been found in samples collected from sixteen Sub-Sahara African countries and China (Afoakwah *et al.,* 2011)

Afoakwah *et al*., 2011 concluded that Single nucleotide polymorphisms of PfATPase6, which have been shown to reduce susceptibility to artemisinins are rarely observed and that their prevalence, nevertheless, may increase under the new selective pressure of ACTs and artemisinin monotherapies.

## 2.7 Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a chain reaction in which One DNA molecule is used to produce two copies, then four, then eight and many more copies of DNA. The polymerase chain reaction (PCR) is perhaps the most powerful laboratory technique ever invented. It's exceptional combination of specificity and sensitivity together with great flexibility has led to a true revolution in genetics. PCR has opened doors to areas hidden to all but a few for most of the history of genetics.

Polymerase chain reaction was first conceptualized and operationalized by Kary Mullis and colleagues at Cetus Corporation in the early 1980's (Morrison, 2010). Within 20 years of its discovery, this sensational technique became the basis for several molecular biology protocols and formed the foundation of the Human Genome Project.

PCR enables the *in vitro* synthesis of nucleic acids through which a DNA segment can be specifically replicated in a semi-conservative way which generally exhibits excellent detection limits(Valones *et al*., 2009)

### 2.7.1 Developments in DNA Structure

In the year 1953, Watson and Crick discovered the double helix structure of the DNA, showing that DNA has two strands with complementary bases running in opposite directions. More importantly, their report mulled over the possibility of a copying mechanism for DNA. Their double helix structure won them the Nobel Prize in 1962(Cheriyedath, 2017)

The first DNA polymerase was identified by Arthur Kornberg in 1957 during his studies on the DNA replication mechanism. This enzyme needed a primer to start copying the template and could create DNA only in one direction. In 1971, Gobind Khorana, a Nobel Prize winner for his part in the Genetic Code discovery, and his team of researchers started working on DNA repairsynthesis. This technique sought to simplify gene synthesis by using artificial

primers and templates that help DNA polymerase to copy the desired gene segments (Cheriyedath, 2017).

### 2.7.2 The PCR Reaction Components

Despite the numerous variations on the basic theme of PCR, the reaction itself is composed of only a few components. These are as stated in the table below, as well as the key steps in PCR

Table 2; PCR components.

| Pcr component | Function/ uses |
|---|---|
| Water | Provides the liquid environment for the reaction to take place |
| PCR Buffer | Provides an optimal pH and monovalent salt environment or the final reaction volume. commercially supplied PCR buffers already contain magnesium chloride($MgCl_2$) |
| $MgCl_2$ | $MgCl_2$ supplies the $Mg^{++}$ divalent cations required as a cofactor for Type II enzymes, which include restriction endonucleases and the polymerases used in PCR. |
| dNTPs | Supplies deoxynucleotide triphosphates (dNTPs) that is individual bases A, T, G, and C, which are the building brick for the double stranded DNA |
| Target DNA | Contains the extracted DNA of interest. Provides a single stranded DNA to be copied during DNA denaturation |
| Primer | Tiny segments of single-stranded DNA that help DNA polymerase to initiate the synthesis of new DNA strands. Primers are complementary to the target |

| | |
|---|---|
| | DNA sequence. |
| Polymerase | polymerase enzyme makes the complimentary copy of the template |

**Table 3: Key Steps in PCR**

| | |
|---|---|
| ***DNADenaturation*** | A high temperature of 95°C is applied to the original double stranded DNA. Heat denatures the DNA by breaking down its weak hydrogen bonds. Thus, the double stranded DNA separates giving rise to single stranded DNA. |
| ***Primer Annealing*** | The denatured DNA is cooled down to 45 - 72°C allowing the primers to bind to their complementary sequence in the single stranded target DNA. Annealing temperature is a crucial factor as the hybridization of DNA is a temperature-dependent process. If this temperature is too high primer-template pairing will not happen, thus hindering the chain reaction. |
| ***Extension of Primer*** | The mixture is then heated to 72°C, at which DNA polymerase starts to add nucleotides to the primer using the target DNA as a template and extends the primer resulting in new DNA formation. |
| ***Repeat Steps*** | The above 3 steps are repeated about 30 times for amplification of the newly formed DNA. For each new cycle, the DNA formed in the previous cycles also serves as a template and therefore the amplification is exponential. |

.

### s2.7.3 Types of PCR

There have been several versions of the standard PCR over time. Some of the key variants of PCR are mentioned below

#### 2.7.3.1 Reverse Transcription PCR (RT-PCR):

These allows the production of DNA templates corresponding to an RNA sample with the help of an enzyme called reverse transcriptase (Cheriyedath, 2017).

Reverse transcription-quantitative PCR (RT-qPCR) has been widely adopted to measure differences in mRNA levels; however, biological and technical variation strongly affects the accuracy of the reported differences(Remans *et al*., 2014). According to Remans and his co-workers, the goal of RT-qPCR experiments is to estimate the true *in-vivo* GOI mRNA levels. To minimize both biological and technical variation and ensure the reliability of RT-qPCR data, they suggested that appropriate reference genes should be selected and assessment of qPCR efficiency should be done.

#### 2.7.3.2 Real Time PCR

This is an advanced variant of PCR where the entire chain reaction is monitored and data is gathered in real time. Real-Time PCR, has become increasingly important in clinical diagnostics and research laboratories due to its capacity for generating quantitative results. This technique allows accompanying the reaction and presentation of results in a faster and more accurate fashion than conventional PCR, which only displays the qualitative results. Real-Time PCR needs a thermocycler with an optical system to capture fluorescence and a computer with software capable of apprehending the data and performing the final analysis of the reaction(Valones et al., 2009).

Fluorescence emissions generates a signal that is directly proportional to the amount of PCR

products. Fluorescence values are recorded during each cycle and represent the amount of amplified product. The fluorescent composites used are *SYBR® Green and* TaqMan (Kubista *et al.,* 2006)

#### 2.7.3.3 *Conventional/ Traditional PCR*

This type of PCR enables the synthesis of specific DNA fragments using a DNA-polymerase enzyme, which takes part in the replication of the cellular genetic material. This enzyme synthesizes a complementary sequence of DNA, using a small fragment (primer) that is attached to one of the DNA strands in the specific site chosen to start the synthesis(Valones *et al.,* 2009). Valones *et al.*2009 claimed that the employment of primers limits the sequence to be replicated and the result is the amplification of a particular DNA sequence with billions of copies

### 2.7.4 Principles and applications in Microbiology:

Conventional PCR has been used for over a period of time in clinical microbiology laboratory research for the identification of microbial pathogens and in pharmaceutical microbiology and biotechnology for detection, molecular identification of drug resistant markers in microorganisms (Wolk *et al.,* 2001). However, for a number of reasons, as reported by Valones *et al.,* 2009, this technique has been restricted to the detection of microorganisms that either have slow growth or cannot be cultivated. Most tests based on conventional PCR involve multiple steps and, therefore, require careful expertise. These assays often require both time and culture-based methods, thereby increasing the costs. Conventional PCR also involves an open-reaction system, which is more susceptible to contamination from foreign amplified DNA.

## 2.8 DNA Sequencing:

The term DNA sequencing refers to methods for determining the order of the nucleotides bases adenine, guanine, cytosine and thymine in a molecule of DNA (Munshi, 2012). Knowledge of DNA sequences has become vital for basic biological research, and has been successfully applied in the following research fields such as diagnostic, biotechnology,forensic biology and biological systematics.

Fifteen years elapsed between the discovery of the DNA double helix in 1953 and the first experimental determination of a DNA sequence by Sanger in 1968.This delay was caused by several factors outlined below:

- The chemical properties of different DNA molecules were so similar that it appeared difficult to separate them.
- The chain length of naturally occurring DNA molecules was much greater than for proteins and made complete sequencing seems unapproachable.
- The 20 amino acid residues found in proteins have widely varying properties that had proven useful in the separation of peptides.
- The existence of only four bases in DNA therefore seemed to make sequencing a more difficult problem for DNA than for protein and
- No base-specific DNAases were known. Protein sequencing had depended upon proteases that cleave adjacent to certain amino acids.

### 2.8.1 DNA sequencing techniques

In 1975, Sanger introduced the plus and minus method for DNA sequencing. This was a critical transition technique leading to the modern generation of methods that have completely dominated sequencing over the past years (Hutchison, 2007). The key to this advance was the use of polyacrylamide gels to separate the products of primed synthesis by DNA polymerase in order of increasing chain length. In 1977Maxam and Gilbert

developed a DNA sequencing method that was similar to the Sanger and Coulson method in using polyacrylamide gels to resolve bands that terminated at each base throughout the target sequence, but very different in the way that products ending in a specific base were generated (Hutchison, 2007). Other advanced method of DNA sequencing followed rapidly after the plus and minus method of singer and the Maxam and Gilbert method.

#### 2.8.1.1 Chain-terminator sequencing method:

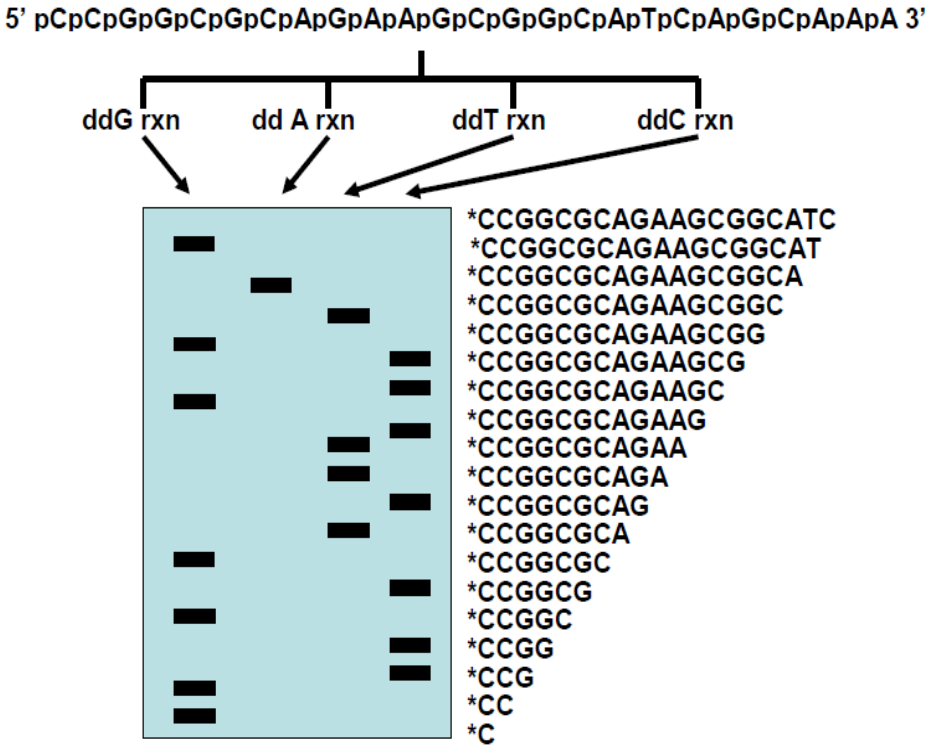
Chain-terminator sequencing also known as Sanger sequencing (after its developer

Frederick Sanger) or dideoxy sequencing, it was more efficient than the previous methods

and used fewer toxic chemicals and lower amounts of radioactivity (Munshi, 2012). The

key principle of the Sanger method is the use of dideoxynucleotide triphosphates

(ddNTPs) as DNA chain terminators. The classical chain-terminator method requires a




single-stranded DNA template, a DNA primer, a DNA polymerase, radioactively or fluorescently labeled nucleotides, and modified nucleotides that terminate DNA strand elongation. The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP) which are the chain-terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length (Munshi, 2012) The newly synthesized and labeled DNA fragments are separated by size by gel electrophoresis on a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, C); the DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image.

Figure 12 : A Sanger sequencing scheme

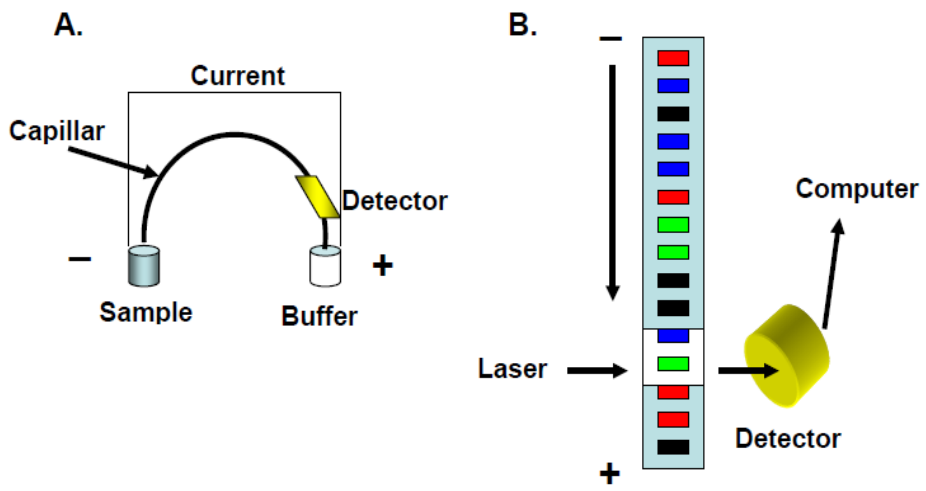
(Integrated DNA Technologies, 2011)

### 2.8.1.2 Automated Fluorescence Sequencing

The most dramatic advance in sequencing was the introduction of automated sequencing using fluorescence-labeled dideoxy-terminators. In 1986, Leroy Hood and colleagues reported on a DNA sequencing method in which the radioactive labels, autoradiography, and manual base calling were all replaced by fluorescent labels, laser induced fluorescence detection, and computerized base calling (Hutchison, 2007). In their method, the primer was labeled with one of four different fluorescent dyes and each was placed in a separate sequencing reaction with one of the four dideoxynucleotides plus all four deoxynucleotides. Once the reactions were complete, the four reactions were pooled and run together in one lane of a polyacrylamide sequencing gel. A four-color laser induced fluorescence detector scanned the gel as the reaction fragments migrated past. The fluorescence signature of each fragment was then sent to a computer where the software was trained to perform base calling. This method was commercialized in 1987 by Applied Biosystems (Hutchison, 2007).

#### 2.8.1.2.1 Steps in Automated Fluorescence DNA sequencing:

DNA sequencing reactions can be carried out in a single reaction tube and be prepared for loading once the reaction reagents had been filtered out. The capillary system is set up to



deliver new polymer to the capillary, load the sequencing reaction into the capillary, apply a constant electrical current through the capillary, and have the resolved fragments migrate past an optical window where a laser would excite the dye terminator, a detector would collect the fluorescence emission wavelengths, and software would interpret the emission wavelengths as nucleotides. It is reported that such systems can deliver 500−1000 bases of high quality DNA sequence in a matter of a few hours (Integrated DNA Technologies, 2011)

Figure 13 : Schematic Representation of a Capillary-based DNA Sequencing System

Source: (Integrated DNA Technologies, 2011)

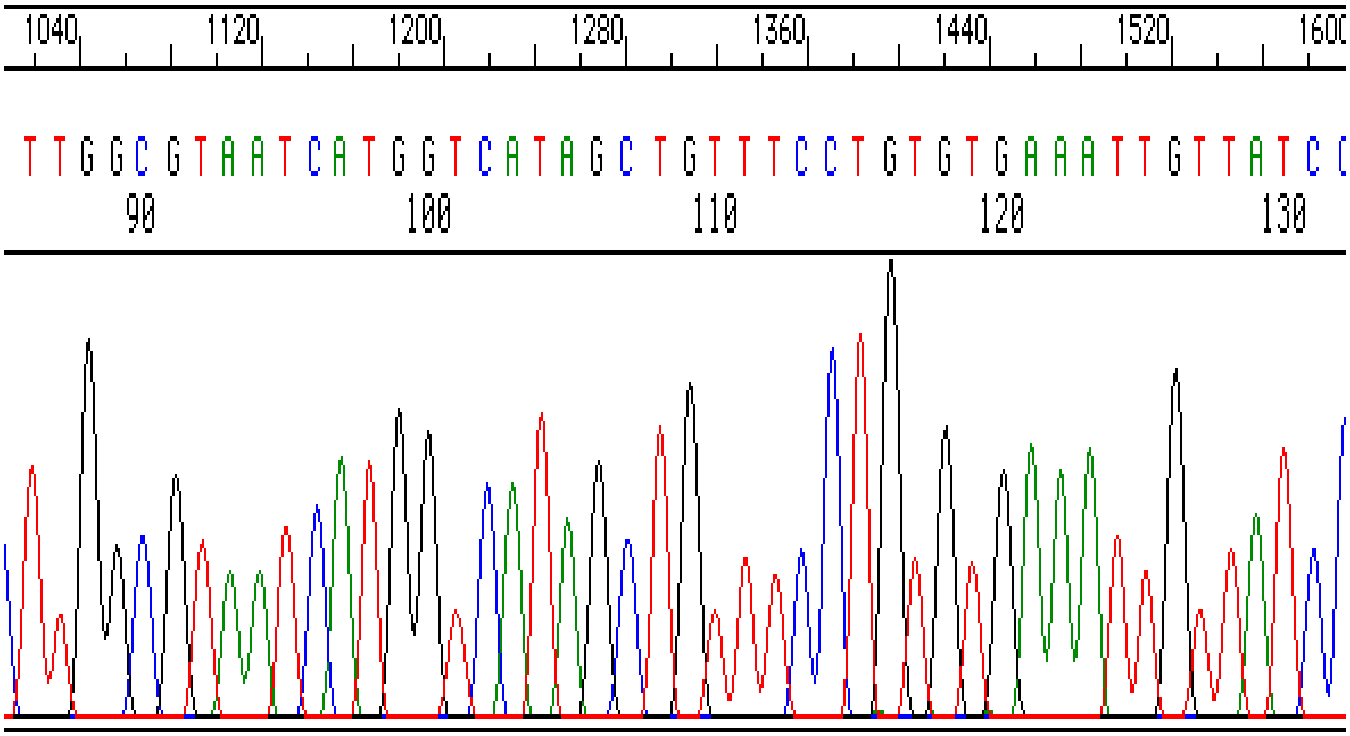



Figure 14: Final sequence electropherogram output.

Source: (Integrated DNA Technologies, 2011)

# CHAPTER THREE:
# METHODOLOGY

## 3.1Study sites

The study was conducted in Nnewi, a town in Anambra State, South Eastern Nigeria. Based on the 2006 national census, Nnewi has a population of about 194,002 people . The town, with an annual rainfall of about 1.4 meters lies mainly in the deciduous forest area which spreads towards the grassland belt, south of Nnewi. Farming is the traditional and major occupation of the people of the town and various food crops including yam, maize, vegetables, fruits and cash crops mainly cocoa and kolanuts are grown. Poultry farming and fish farming are also undertaken. Also, some of them also involve themselves in trading particularly motorcycle spare parts. Malaria is present throughout the year with a marked increase during the raining season which normally runs from April to September in the town.

## 3.2 Sample Size Determination

The sample size (n) were determined using single proportion formula (Onwuasigwe *et al*., 2004)

$$n = \frac{Z^2P}{d}$$

For a population greater than 10,000

Where:

n = Minimum Sample Size.

Z = standard normal deviation usually set at 1.96 which correspond to 95% confidence level (obtained from a statistical table). Therefore $Z_{0.95} = 1.645$

P = Prevalence rate. Using a 70.5 % prevalence of malaria

Then p = 0.114

q = 1 – p, if p = 0.71, then q will be 1- 0.71 = 0.29

d = degree of precision desired, (that is closeness of the sample estimate to the actual population) set at 0.05 (using 95% confidence level).

$$n = \frac{1.645^2 \times 0.71 \times 0.29}{0.05^2} = 222.86$$

However 10% of the calculated sample size is added to each of the isolate to allow for attrition.

222.89+ 22.286=245.176approximately 245.

The minimum sample size were 245 samples.

### 3.3 Study Participants

Three cohorts were surveyed for Malaria parasites: Children (≤10 years),Pregnant women attending antenatal clinic and other adult cohort. All were asymptomatic as inclusion criteria defined could accommodate only participants without obvious symptoms of malaria (axillary temperature ≥ $37.5^0$C or history of fever 72h preceding presentation). Other enrolment criteria include absence of severe illness, a written consent from participants or guardians, and assent in cases where participants were children.

### 3.4 Ethical Issues:

Ethical approval for the study was granted by the Ethics Review Board, University of Nigeria Teaching Hospital, Enugu, South Eastern Nigeria. Informed consent was also obtained from parent or guardian of each child prior to being included in the study.

### 3.5 Sample Collection and Microscopy

Blood was collected by venepuncture from each participants for parasitological and molecular analysis. Six drops of blood were spotted on filter paper, air dried, individually sealed and stored at room temperature until DNA extraction. Thick and thin blood smears were labelled and allowed to dry. Slides were stained with freshly prepared Giemsa stain. The blood films were examined for malaria parasites. Parasites were quantified relative to 250 white blood cells and estimated as parasites per microlitre assuming a mean WBC of 8000/ul of blood. Blood smears were labelled negative if no parasites were seen after examination of 200 oil immersion fields (x 1000) on a thick blood film.

### 3.6 DNA Extraction

DNA was extracted from the dried blood spots on filter paper using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and stored in -20$^{o}$C until further use. Briefly, 3 punched- out circles from a dried blood spot were placed into a 1.5 ml microcentrifuge tube. 180 ul of ATL Buffer was added, incubated at 85$^{o}$C for 10 minutes. 20 ul of Proteinase K was added, mixed by vortexing and incubated at 56$^{0}$C for 1 hour. The 1.5 ml microcentrifuge tube was centrifuged briefly, followed by addition of 200 ul of AL (incubated at 70$^{o}$C for 10 min). 200 ul of ethanol (96-100%) was added to the mixture. The resultant mixture was then applied to the QlAamp Spin column without wetting the rim. The column was centrifuged at 6000 x g (8000 rpm) for 1 min. The QlAamp Spin Column was placed in a 2ml collection tube. 500 ul Buffer AW1 was added without wetting the rim. This was followed by centrifugation at 6000 x g (8000 rpm) for 1 min. QlAamp spin column was placed again in another clean 2ml collection tube and the filtrate discarded. Another AW2 buffer was added (500 ul). Now, centrifugation was done at full speed of 20,000 x g for 3 min. The filtrate was discarded and the QlAamp Spin Column placed in a clean 1.5 ml

microcentrifuged tube. 200 ul buffer AE was added, followed by final centrifugation at 6000 x g for 1 min. The filtrate was stored as the DNA for further analysis.

### 3.7 Detection and Identification of *Plasmodium* Species

For the detection and identification of *Plasmodium* species we optimized the nested-PCR method described asNP-2013 protocol, an update for the widely usedNP-1993 to NP-2005 (SSU rRNA) protocols forall human malaria parasites molecular detection(Zeeshan*et al*.,2012). For the *Plasmodium* genus detection a firstPCR was done with specific primers-rPLU1 andrPLU5 (Snounou*et al*., 2002)followed by genus specificnested PCRtospecies level in 4separate reactions as reported previously:rFAL1/rFAL2 - *P. falciparum*(Snounou*et al*., 2002); rVIV1/rVIV2 - *P. vivax* (Snounou et al., 2002); rMAL1/ rMAL2 –*P. malariae* (Snounou*et al*., 2002);and rOVA1WC/rOVA2WC - *P. ovale* (Fuehrer*et al*., 2012) (Table 1), using the PCRamplification of the first reaction (rPLU1 andrPLU5) as a DNA template.All PCR was performed in 25 µl total volume,containing 1X buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 200 nM primers, and 1U Taq DNA-polymerase (Qiagen,Hilden, Germany). The additionally added MgCl2and primers varied as detailed in Table 2. Thefirst PCR round used 5 µl of extracted DNA fromthe blood spotted on filter paper samples. Thesecond PCR reactions used 2 µl of the PCRproduct obtained for the first PCR. Theamplification conditions for all PCR reactions areshown in Table 1.Thermal cycling was performed in PTC-200 Thermal cycler (MJ Research, USA). Eachamplification run included one negative control( a negative control of DNAextract) and one positive control ( Laboratory adapted 3D7 for *P.falciparium* and DNAextracted from positive clinical samples of *P. Vivax*)). For nested-PCR reactions, anadditional negative control was added, consistingof 2 µl of the negative control reaction of the first run of PCR.

### 3.8 Visualization

Amplicons were separated on a 1.5% agarose gel electrophoresis run along with a 100 bp DNA ladder (Invitrogen, Karlsruhe, Germany). Gels were stained with CYBR$^{R}$ GREEN 1 nucleic acid gel stain (Cambrex Biosciences, East Rutherford, NJ) and visualized on a dark reader transilluminator (Clare Chemical Research, Dolores, CO). The presence or absence of different *Plasmodium* species was confirmed with representative amplicon size that were species-specific. Samples that failed to amplify were subjected to repeated amplification procedures with different PCR additives.

Table 4: **PCR primers and Conditions employed in *Plasmodium species* Identification**

| Species/genus | Primer name | PCR primers (5'-3') | Size(bp) | Amplification conditions |
|---|---|---|---|---|
| Genus_nested 1 | rPLU1 | TCAAAGATTAAGCCATGCAAGTGA | ~1,670 | Initial denaturation, 95ºC for 5 mi |
| | rPLU5 | CCTGTTGTTGCCTTAAACTTC | | cycles of 94ºC for 1 min, 58ºC fo min, and 72ºC for 2 min; final extension, 72ºC for 5 min |
| *P. falciparum* | rFAL1 | TTAAACTGGTTTGGGAAAACCAAATATATT | 206 | Initial denaturation, 95ºC for 5 n |
| | rFAL2 | ACACAATGAACTCAATCATGACTACCCGTC | | cycles of 94ºC for 1 min, 58ºC min, and 72ºC for 2 min; |
| *P. malariae* | rMAL1 | ATAACATAGTTGTACGTTAAGAATAACCGC | 145 | final extension, 72ºC for 5 |
| | rMAL2 | AAAATTCCCATGCATAAAAAATTATACAAA | | |
| *P. vivax* | rVIV1 | CGCTTCTAGCTT AATCCACATAAC TGATAC | 121 | |
| | rVIV2 | ACTTCCAAGCCG AAGCAAAGAAAG TCCTTA | | |
| *P. ovale* | rOVA1 | TGTAGTATTCAA ACGCAGT | 659-662 | |
| | rOVA2 | TATGTACTTGTTAAGCCTTT | | |

### 3.9 Detection of *Plasmodium vivax*by Real-time qPCR assays

Quantitative real-time PCR specifically the SYBR Green detection method was employed (Rougemont*et al.*, 2004) using *P. vivax*specific primers (forward: 5'-GAATTTTCTCTTCGGAGTTTATTCTTAGATTGC-3'; reverse: 5'GCCGCAAG CTCCACGCCTGGTGGTGC-3') that targeted on the plasmodial 18S rRNA region (Lo *et al*., 2016). Amplification was done in a 10µl reaction mixture containing 2 µl of genomic DNA, 5 µl of 2 x SYBR Green Qpcr Master Mix ( Thermo Scientific), and 0.5 µM primer. Reaction was performed in CFX96 Touch ™Real – Time PCR Detection System (BIORAD), with an initial denaturation at $95^0$C for 3 min, followed by 45 cycles at $94^0$C for 30 secs, $55^o$ C for 30secs, and 68 $^o$C for 1 minute with final 95 $^o$C for 10 secs. Amplification curve of both positive, negative and of the various isolates were observed on the screen of the q PCR machine.

**3.10 Duffy Genotyping:**

This was done to check DARC gene polymorphisms using nested PCR procedure previously reported with slight modification (Menard*et al*., 2010). Duffy genotyping included the GATA-1 transcriptionfactor binding site at nucleotide position −33 (t, wildtype; c, erythrocyte silent), the Fya/Fyb antigen site at codon 42(ggt encodes G, $Fy^a$; gat encodes D, $Fy^b$), and the $Fy^{bweak}$ antigensite at codon 89 (cgc encodes R, $Fy^b$; tgc encodes C, $Fyb^{weak}$).Primary PCR amplifications were performed in a reaction mixture (25µL) containing 1.5 µL DNA, 0.4 µM each primer(**DF1**,5-GTGGGGTAAGGCTTCCTGAT-3;**DF2**,5'CAGAGCTGCGAGTGCTACCT-3′ ), 250 µMeach dNTP, 2.5 mM MgCl2, and 1.25 units DNA Polymerase under the following conditions:94 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for30 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min (PCRproduct 997 bp).

Nested PCR amplifications for SNP detection in the GATA-1transcription factor binding site (**DF1**,5′ -GTGGGGTAAGGCTTCCTGAT-3′ ;**DF4**,5'CAAACAGCAGGGGAAATGAG-3′ ) and exon 2 codon region (forward primer,5′ -CTTCCGGTGTAACTCTGATGG-3′ ; reverse primer, 5′ -CAGAGCTGCGAGTGCTACCT-3′ ) were performed in separatereaction mixtures (25 µL) with 1.5 µL of PCR products, 0.36 µMeach primer, 250 µM each dNTP, 2.5 mM $MgCl_2$, and 1.25 units DNA Polymerase following the amplification conditionsprovided above for 30 cycles [PCR products, 223 bp(GATA-1) and 402 bp (exon 2)].

### 3.11Genotyping for Msp 1, Msp2 and Glurp

#### 3.11.1Merozoite Surface Protein 1

The highly polymorphic locus, msp-1 block 2, was used for the genotyping of the *P. falciparum* population using the nested polymerase chain reactions technique. The msp-1 primers sequences used in this studyare shown in Table 5 (Ahmedou Salem*et al*., 2014). Primer pairs corresponding to the flanking sequence of the conserved regions of MSP- 1 was used for the primary amplification (outer region). The second amplification reaction (nested PCR) were based on the primary products using allelic specific primers sets coresponding to K1, RO33, and Mad20 families of msp-1. All outer and nested PCR reactions were done in 20 ul final volume containing 1X buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 200 nM primers, and 1U Taq DNA-polymerase with approximately 10 ng of DNA template on a PTC-200 Thermal cycler (MJ Research,Watertown, USA) using the following cycling conditions: initial denaturation for 3 min at 94°C, followed by 30 cycles of 25 s denaturation at 94°C, 35 s annealing at 50°C, and 2 min 30 extension at 68°C. Final extension was carried out at 72°C for 3 min. Amplicons were separated on a 1.5% agarose gel electrophoresis run along with a 100 bp DNA ladder (Invitrogen, Karlsruhe, Germany). Gels were stained with CYBR$^R$

GREEN 1 nucleic acid gel stain (Cambrex Biosciences, East Rutherford, NJ) and visualized on a dark reader transilluminator ( Clare Chemical Research, Dolores, CO).

Table 5:**Sequences of oligonucleotide primers used to genotype msp-1 block 2 allelic types of *Plasmodium falciparum* isolates from Nnewi, Nigeria**

| Amplification Primer | Sequences | Polymorphism |
|---|---|---|
| Outer PCR | | |
| Msp1 F | 5′AAGCCTTAGAAGATGCAGTATTGAC3′ | Conserved |
| Msp1 R | 5′ATTCATTAATTTCTTCATATCCATTATC3′ | |
| Nested PCR | | |
| K1F | 5′AAGAAATTACTACAAAAGGTG3′ | K1 family specfic |
| K1R | 5’TGCATCAGCTGGAGGGCTTGCACCAC3′ | K1 family specfic |
| RO33 F | 5′AGGATTTGCAGCACCTGGAGATCT3′ | RO33 family specific |
| RO33 R | 5′GAGCAAATACTCAAGTTGTTGCA3′ | RO33 family specfic |
| Mad20 F | 5′TGAATTATCTGAAGGATTTGTACGTC3′ | Mad20 family specific |
| Mad 20 R | 5′GAACAAGTCGAACAGCTGTTA3′ | Mad20 family specific |

### 3.11.2 Merozoite Surface Protein 2

Nested PCR was used to genotype the highly polymorphic region of Msp-2 (block 3). Set of primers used are in table 3 (Felger *et al*., 1999). The PCR reaction mixture is as that of Msp-1 (block 2) with slight modification in the cycling parameters on a PTC-200 Thermal cycler (MJ Research,Watertown, USA) using the following cycling conditions:initial denaturation at $94^0$C for 5 minutes, followed by 30 cycles at $94^0$C for 10 seconds; $57^0$C for 30 seconds, and $72^0$C for 40 seconds. The final cycle had a prolonged extension at $72^0$C for 3 minutes. Amplicons were separated on a 1.5% agarose gel electrophoresis run along with a 100 bp DNA ladder (Invitrogen, Karlsruhe, Germany). Gels were stained with CYBR$^R$ GREEN 1 nucleic acid gel stain (Cambrex Biosciences, East Rutherford, NJ) and visualized on a dark reader transilluminator ( Clare Chemical Research, Dolores, CO).

**Table 6: Primer Sequences used for Msp-2 Genotyping**

| Locus | Primer | Primer Sequence |
|---|---|---|
| Primary PCR | | |
| MSA 2 | MSA 2-1 | 5'-ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3' |
| | MSA 2-4 | 5'-TTA TAT GAA TAT GGC AAA AGA TAA AAC AAG-3' |
| Secondary PCR | FC 27-1 | 5'-GCA AAT GAA GGT TCT AAT ACT AAT AG-3' |
| | FC 27-2 | 5'-GCT TTG GGT CCT TCT TCA GTT GAT TC-3' |
| | 3D7-1 | 5'-GCA GAA AGT AAG CCT TCT ACT GGT GCT-3' |
| | 3D7-2 | 5'-GAT TTG TTT CGG CAT TAT TAT GA-3' |

### 3.11.3 Glutamate rich Protein

*P. falciparum* were genotyped for polymorphic GLURP R2 repeat region by semi – nested PCR reaction using the following set of primers (Snounou *et al*., 2002):

GOF: 5′TGAATTTGAAGATGTTCACACTGAAC3′;

GOR: 5′GTGGAATTGCTTTTTCTTCAACACTA A3′ and

GNF: 5′TGTTCACACTGAACAATTAGATTTAGA TCA 3′. In brief, both the primary and semi-nested amplification were carried out in a 20 ul reaction volume containing 1X buffer, 1.5 mM $Mgcl_2$, 200 µM dNTPs, 200 nM primers, and 1 U Taq DNA- polymerase with approximately 10 ng of DNA template on a PTC-200 Thermal cycler (MJ Research, USA). The thermal cycling parameters for the first round were: initial denaturation at 95$^{o}$C for 5 min, followed by 30 cycles of 94°C for 30 seconds for denaturation, I min for 54°C for annealing temperature, and60 sec at 72°C extension and the thermal parameters forthe second PCR were followed by 30 cycles of, respectively, 30sec at 94°C for denaturation, 60 sec for

59°C for annealingtemperature, and 60 sec at 72°C extension. Thiswas followed by a final extension of 5 min at 72°C. Electrophorosis and Visualization were done as stated in MSP 1 and 2 above.

### 3.12 Genotyping for *Plasmodium falciparum*Circumsporozoite Protein

This was done by using protocol already described somewhere (Zeeshan*et al*., 2012). Briefly,theCSP1forward (5'-TTAGCTATTTTATCTGTTTCTTCC-3')and CSP2 reverse (5'-TAAGGAACAAGAAGGATAATACC-3') primers designed using 3D7 strain as a reference sequence,were used to amplify 1177 bp of the 1194 bp complete pfcsp gene (Zeeshan*et al*., 2012) .The PCR cycling conditions for this primer pair were: 10 minutesinitial denaturation at $94^0$C followed by 35 cycles with 1 minutedenaturation at $94^0$C, 1 minute annealing at $57^0$C, 90 secondsextension at $72^0$C and a final 10 minute extension at $72^0$C. Theresulting PCR products were diluted 1:10 and 2 ml of this was usedas a template to amplify the internal 1026 bp fragment usingCSP3 forward (5'-GAAATGAATTATTATGGGAAACAG-3')and CSP4 reverse (5'-GAAGGATAATACCATTATTAATCC-3') primers. The 1026 bp fragment encompassed the N-terminal Tcell epitope, central repeat and C-terminal T cell epitope regions.The PCR cycling conditions for CSP3/CSP4 primer pair were:10 minutes initial denaturation at $94^0$C followed by 35 cycles with1 minute denaturation at $94^0$C, 40 seconds annealing at $57^0$C,80 seconds extension at $72^0$C and a final 10 minute extension at$72^0$C. A negative control without thetemplate DNA was always used to avoid cross contamination. Further, the DNA from a cultureadapted *P.falciparum* was used as a control to check the reliability of the amplification. The amplified products were resolved on 1.5% agarose gel as stated above.

### 3.13 Sequencing of the amplified CSP products

The methods for cyclesequencing PCR and cleanup were same as described earlier (Vinayak *et al*., 2006). Briefly, PCR product were first cleansed using Exo-SAP-IT ( USB, Affymetrix, USA), and 1 ul of the purified product was used as a template for direct sequencing using Big Dye terminator v. 2.0 cycle sequencer, according to manufacturer's instruction. The products were sequenced on both strands using CSP3forward, CSP4 reverse and CSP-D reverse (5'-TGGGTCATTTGGCATATTGTG-3') primers, using ABI BigDye Terminator Ready Reaction Kit Version 3.1 (PE AppliedBiosystems, CA, USA) on an ABI-310 genetic analyzer (ABI 310Genetic Analyzer; PE Applied Biosystems, CA, USA).

### 3.14 Multiplicity of infection

As already described somewhere (Oyebola*et al*., 2014), the multiplicity of infection (MOI) or number of genotypes per infection was estimated by dividing the total number of fragments detected in one antigenic marker by the number of samples positive for the same marker. The mean MOIwas calculated by dividing the total number of fragmentsdetected in both msp1, msp2 and glurp loci by the number ofsamples positive for both markers. Isolates with more than one allelic family were considered as polyinfections while the presence of a single allelic family was considered as monoinfection. Samples possessing only one genotype per allelic family were monoclonal while possession of multiplegenotypes per family was described as polyclonality.

### 3.15 Genotyping for DHPS and DHFR Mutations

Mutations in the DHPS and DHFR were assessed by a protocol already descsribed by Woldearegal *et al*. in 2005 with slight modification using nested PCR and sequencing to detect muttions in the DHFR and DHPS genes. Two microlitre(2 µL) of DNA was used as a

template DNA in a 20 µL PCR that contained 0.4 µL of each oligonucleotide primer, 1 x PCR buffer (Qiagen), 0.2 mM dNTPs, 2.0 mM Q solution and 0.08 units/ µL of Tag polymerase. The primary reaction was to amplify 665- basepair (bp) portion of the DHFR gene using primers Amp1 ((5'TTTATA TTT TCT CCT TTT TAT-3') and Amp 22 ((5'-TTACTA GTA TAT ACA TCG CTA ACA G-3'). Also, a 727-bp portion of the DHPS gene was amplified by using primers sulf5 (5'-GGT ATT TTT GTT GAA CCT AAACG-3') and sulf3'((5'-TCC AAT TGT GTG ATT TGT CCAC-3'). 3 ul of the primary PCR was used as a template in the nested PCR of the DHFR gene. Oligonucleotide primer SP1 ( 5'- ATG ATG GAA CAA GTC TGC GAC-3') and Amp22 were used to amplify a 646-bp fragment containing codons 16, 51, 59, 108, and 164. Using the same similar reaction conditions like those used for DHFR, DHPS was nested using a different set of primers sulf5' and Leo2 (5'-CTG GAT TAT TTG TAC AAG CAC-3') to amplify a 319-bp fragment of the DHPS gene having the sequences of codons 436 and 437. In addition, a 472-bp DHPS fragment that covers 540, 581, and 618 was amplified using primer pair DS-5F (5'-GAA TGT GTT GAT AAT GAT TTA G-3') and sulf3'. Visualization and electrophoresis were done as described above.

### 3.16 Detection of *Pfcrt,Pfmdr1, Pfatpase6* and *Pfkelch* 13 Polymorphisms.

Apart from Pfatpase6 which were genotyped using direct nesting on the extracted DNA, others were amplified using the conventional nested PCR in a 20 µL reaction volume containing 1X buffer, 2.5 mM $Mgcl_2$, 200 µM dNTPs, 2.0 mM Q solutions, 200 nM primers, 1 U Taq DNA- Polymerase and 10 ng of DNA template on a PTC-200 Thermal cycler (MJ Research, USA). The cycling parameters,various primers used, and the amplified codons were listed in Table 5. Visualization of amplified product, purification and sequencing were done as already descibed in this work.

**Table 7: Primer pairs used to amplify and sequence *Pfcrt, Pfmdr1, Pfatpase6 and Pfkelch* 13**

| Gene | Primer | Primer Sequence | PCR Cycling conditions | References |
|---|---|---|---|---|
| Pfcrt SNPs Codon 72 & 76 | OF P1 | 5'-GACGAGCGTTATAGAGAATTA-3' | 35 cycles of 94°C for 30 s; 56°C for 30 s; and 62°C for 1 min; | Mekonnen *et al*. 2014 |
| | OR P2 | 5'-CCAGTAGTTCTTGTAAGACC-3' | | |
| | NF P3 | 5'-GGCTCACGTTTAGGTGGA-3' | 30 cycles of 94°C for 30 s; 56°C for 30 s; and 65°C for 1 min; | Mekonnen *et al*. 2014 |
| | NR P4 | 5'-TGAATTTCCCTTTTTATTTCCAAA-3' | | |
| Pfmdr1 SNPs at Codon 86&184 | OF P5 | 5′-AGGTTGAAAAAGAGTTGAAC-3' | 30 cycles of 94°C for 30 s; 55°C for 30 s; and 65°C for 1 min; | Mekonnen *et al*. 2014 |
| | OR P6 | 5′-ATGACACCACAAACATAAAT-3' | | |
| | NF P7 | 5′-ACAAAAAGAGTACCGCTGAAT-3' | 30 cycles of 94°C 30 s; 60°C | Mekonnen *et al*. 2014 |
| | NR P8 | 5′-AAACGCAAGTAATACATAAAGTC-3' | for 30 s; and 65°C for 1 min | |
| Pfmdr1 SNPs at Codon 1034 1042 and 1246 | OF P9 | 5′-GTGTATTTGCTGTAAGAGCT-3' | 34 cycles of 94°C for 30 s; 55°C for 1 min and 72°C for 1.5 | Mekonnen *et al*. 2014 |
| | OR P10 | 5′-GACATATTAAATAACATGGGTTC | | |
| | NF P11 | 5′ CAGATGATGAAATGTTTAAAGATC | 29 cycles of 94°C for 30 s; 60°C for 30 s; and 65°C for 1 min; | Mekonnen *et al*. 2014 |
| | NR P12 | 5′-TAAATAACATGGGTTCTTGACT | | |
| Pfkelch 13 SNPs at varied codons | O13-1 | 5'-GGGAATCTGGTGGTAACAGC-3' | 30 cycles of 30 secs at 95°C, 2 min at 58°C, 2 min at 72°C | Ariey *et al*. 2014 |
| | O13-4 | 5'-CGGAGTGACCAAATCTGGGA-3' | | |
| | N 13-2 | 5'-GCCTTGTTGAAAGAAGCAGA-3' | 40 cycles of 30 sec at 95°C, 1 min at 60°C, 1 min at 72°C | Ariey *et al*. 2014 |
| | N 13-3 | 5'-GCCAAGCTGCCATTCATTTG-3' | | |
| Pfatapase6 SNPs at codon 241-431 and codon 623 - 769 | NF 1 | 5'- TCATCTACCGCTATTGTATG - 3' | 40 cycles of 45 sec at 94°C, 1 min at 55°C, 1 min at 72°C | Zakeri et al. 2012 |
| | NR 1 | 5' - TCCTCTTAGCACCACTCC - 3' | | |
| | NF 2 | 5'- TGGAGACAGTACCGAATTAGC - 3' | 40 cycles of 45 sec at 94°C, 1 min at 55°C, 1 min at 72°C | Zakeri *et al*. 2012 |
| | NR 2 | 5' - TCTTCCTACATATTTACGTGGTG - 3' | | |

### 3.17 Purification of DNA from TBE Agarose Gels

This was done to further acess the diversity of the *Plasmodium falciparum* of the 3D7 families. Samples at the same band size were cut and DNA purified from the agarose gel using GE Healthcare illustra™ GFX™ PCR DNA and Gel Band Purification Kit. The manufacturers protocol was followed. Briefly, a Dnase –free 1.5 ml microcentrifuge tube was weighed and recorded. Using a clean scapel, long wavelength (365nm) ultraviolet light and minimal exposure time, agarose band containing the sample of interest were cut and placed into the Dnase-free 1.5 microcentrifuge tube (eppendorf). The microcentrifuge plus agarose band were weighed to calculate the weight of the agarose slice. Three hundred microlitre(300 µL) capture buffer type 3 were added. The gel and the buffer were mixed by inverting the tube . Incubation was done at 60 $^{0}$Cfor 15 – 30 minutes until the agarose dissolved completely (the tube were mixed by inversion every 3 minutes in the course of the incubation). The capture buffer type 3- sample mix were centrifuged to collect the liquid at the bottom of the tube. Eight hundred microlitre (800 µL) of capture buffer type 3- sample mix were transfered into the assembled GFX MicroSpin column and collection tube. This was followed by incubation at room temperature for 1 minute and centrifugation at 16 000 x g for 30 seconds. The flow through was discarded. The GFX MicroSpin column was placed back inside the collection tube. This was done for all the samples. 500 µL wash buffer type 1 to the GFX MicroSpin column and assembled column centrifuged at 16 000 x g for 30 seconds. Collection tube was discarded and GFX MicroSpin transfered to a fresh Dnase- free 1.5 ml microcentrifuge tube (eppendorf). A 20 µL Elution buffer was introduced into the center of the membrance in the assembled GFX MicroSpin column and sample collection tube. This was followed by incubation at room temperature for 1 minute and centrifugation at 16, 000 x g for 1 minute to recover the purified DNA. The purified DNA were stored at -20 until further use.

### 3.18 Sequencing

All the PCR products that were sequenced were first cleansed using Exo-SAP-IT ( USB, Affymetrix, USA), and 1 ul of the purified product was used as a template for direct sequencing using Big Dye terminator v. 2.0 cycle sequencer, according to manufacturer's instruction (Humphreys *et al*., 2007)

### 3.19 Sequences and Statistical Analysis

Data were analyzed using GraphPad Prism 6 (Version 6.0). For descriptive analysis, proportion was used to present the distribution of different allellic families while mean was used to present parasitaemia and MOI. A *P* value of $\leq 0.05$ was considered to be statistically significant different. Allignments of sequences were done using Geneious software ( version 9.1.5 ) and Sequencer ( Demo version 4) for SNPs detection.

# CHAPTER FOUR:

# RESULTS

## 4.1 Children Population Cohorts

### 4.1.1 Demographic characteristics of the Children subjects

Table 8 detailed the characteristics of the study population ( Children). The mean age of the population was 3.1691 ± 0.1704. The age range was from 0.16 years to 10 years. Sex ratio was found to be 1.73 (159:92). Mean parasitaemia and parasite density range were 2078.01 ± 67.73 and 80 to 6240 respectively.

### 4.1.2Mean Parasite density Amongst Children Cohort with respect to Age

Mean parasite density were correlated with age of the population using Anova, Brown – Forsythe test and Bartlett's test ($P \leq 0.05$). The age ranges compared were 0 – 1, 2 – 5 and 6 – 10. The ranges were chosen with understanding of the development of antibodies as one advances in age. The mean parasite densities for the 0 -1, 2 -5 and 6 – 10 ranges are 1811.22 ± 86.60 P/µL, 2169.23 ± 105.52 P/µL and 2417.96 ± 193.92 P/µL respectively (Figure 15). The analysis also revealed that the values were statistically significant. *P* values were 0.0026, 0.0077 and 0.0007 for Anova, Brown – Forsythe test and Barlett's test respectively.

### 4.1.3 Percentage Use of Mosquito Nets Amongst the Children Population

The use of mosquito net was assesed amongst the Children cohort in Nnewi, South East Nigeria. Figure 16 below detailed the percentage use of Mosquito nets as observed in the children population. 34.8% of the population said 'Always' in response to the use of mosquito nets. 59.6 % said 'Never' in response to the use of Mosquito nets, while 5.6 % said that they do use the net 'Sometimes'.

**Table 8: Demographic characteristics of the Children subjects(n=251)**

| Characteristics of Patients | Values |
|---|---|
| Mean age | 3.1691 ± 0.1704 |
| Age range | 0.16 years to 10 years |
| Sex ratio (M:F) | 1.73 ( 159:92) |
| Geometric mean parasitaemia | 2078.01 ± 67.73 |
| Parasite density range | 80 to 6240 |

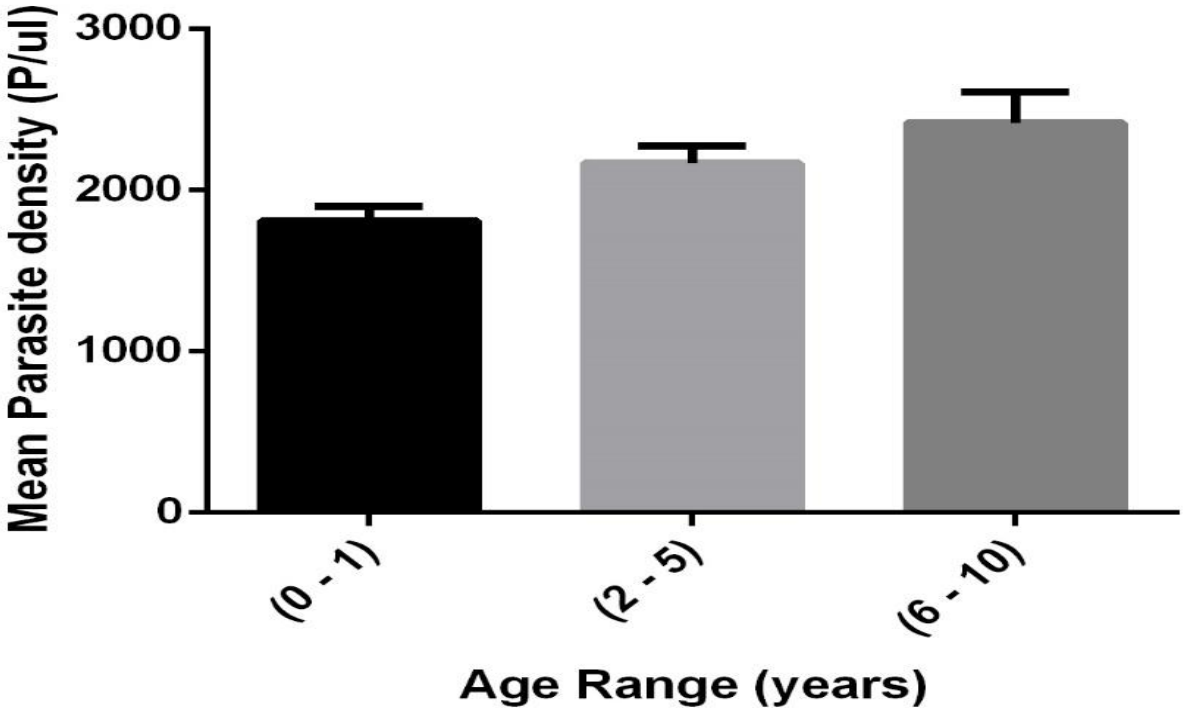



**Figure 15: Mean Parasite density Amongst Children Cohort with respect to Age**

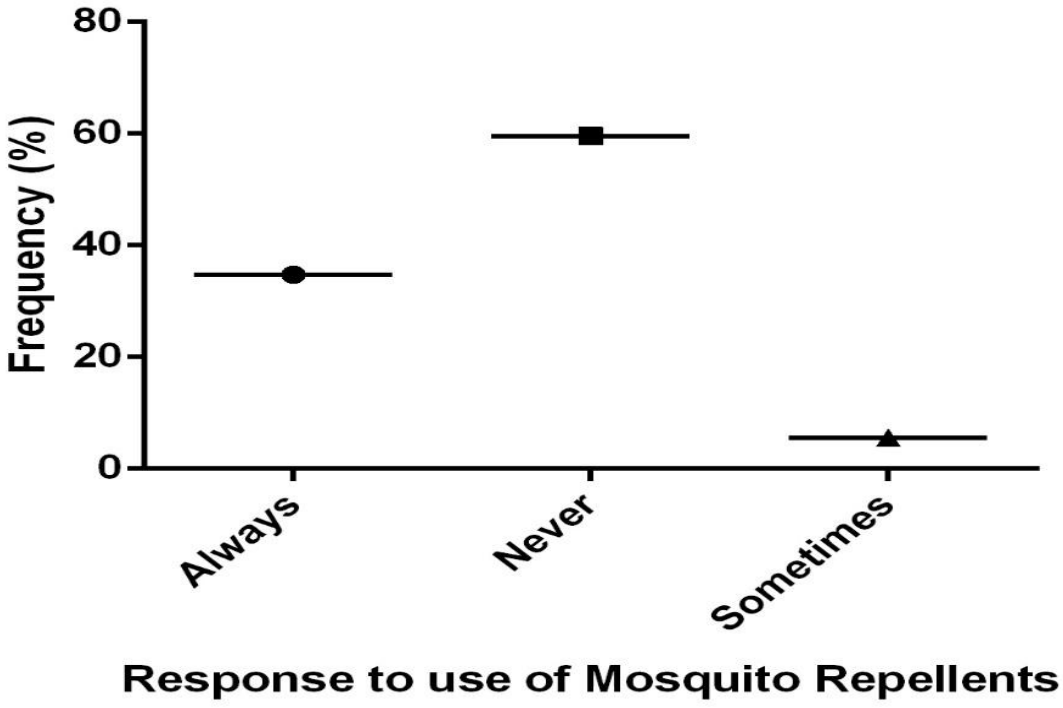



**Figure 16: Percentage Use of Mosquito Nets Amongst the Children Population**

### 4.1.4Mean Parasite Density Amongst Children Population with respect to use of Mosquito Net

Mean parasite density was correlated with the use of mosquito net as shown in Figure 17 below. Anova, Brown – Forsythe and Bartlett's tests were used to compare the means of the parasite density of the various response groups in the population. *P* value was set at $p \leq 0.05$. The mean parasite density values of the various response groups were 2089.767 ± 123.674 (Always), 2178.462 ± 334.0694 (Sometimes) and 2230.693 ± 107.795 (Never). The analysis using the three test approches were statistically significant (Anova $p = 0.0002$, Brown – Forsythe $p = 0.0002$ and Bartlett's tests $p = 0.0001$).

### 4.1.5Percentage Use of Mosquito Repellant Amongst the Children Population in Nnewi, South East Nigeria.

Use of Mosquito repellant amongst the Children Population was assessed. Figure 18 presented the outcome in percentage. 18 % of the population uses mosquito repellent (Aways). About 68.4 % of the population appear not to be using mosquito repellent (Never), while 13.6 % uses mosquito repellent 'sometimes'.

### 4.1.6Parasite Density Amongst the Children Population with respect to use of Repellent

Mean parasite density was compared with the various responds groups in the use of mosquito repellent (Figure 19). Anova, Brown – Forsythe and Bartlett's tests were used for the analysis. P value was set at $p \leq 0.05$. It was observed that response group that said 'Always' had 2367.826 ± 209.227 Mean parasite density (P/µL). The response group that said 'Never'had 2071.111 ± 153.777 mean parasite density (P/µL), while the response group that

said ‘Sometimes’ had 2220.396 ± 107.379 mean parasite density (P/µL). The analysis showed that the three tests used to compare the means of the different response groups had *p* values less than 0.05 ( Statistically significant).

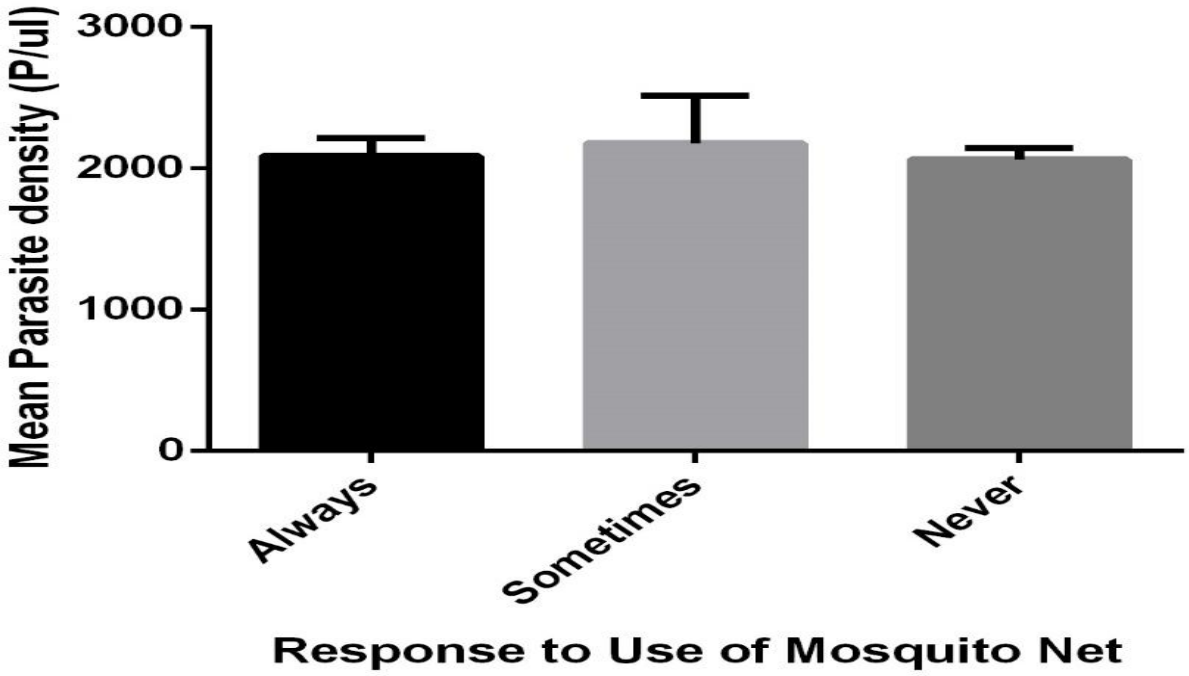



**Figure 17: Mean Parasite Density Amongst Children Population with respect to use of Mosquito Net**

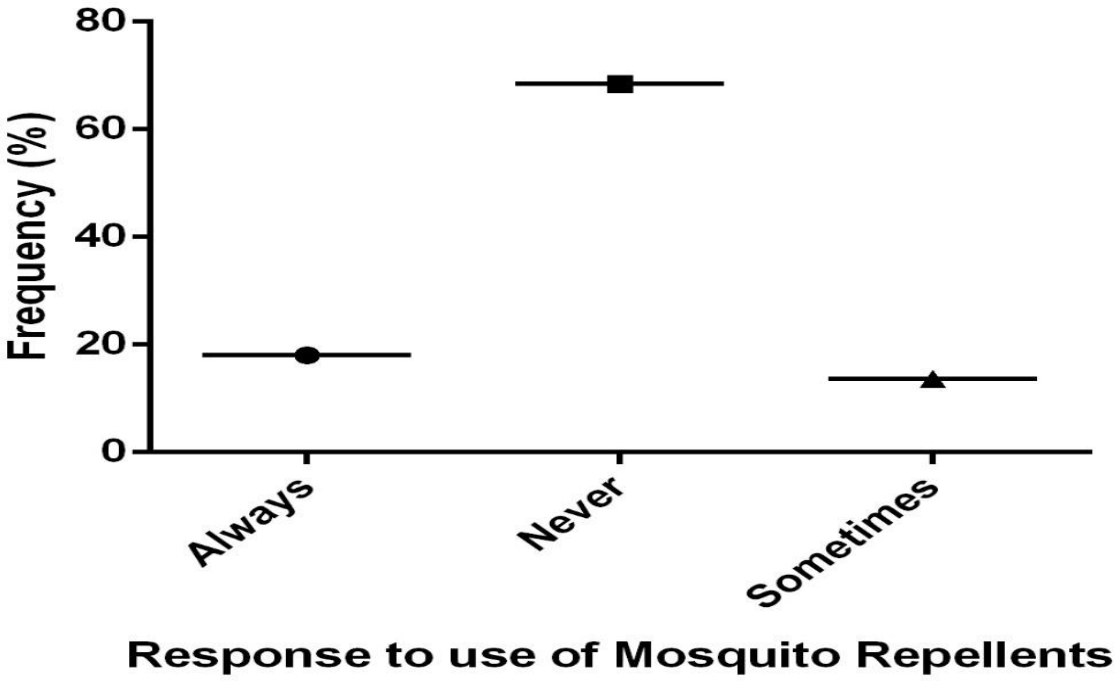



**Figure 18: Percentage Use of Mosquito Repellant Amongst the Children Population in Nnewi, South East Nigeria**.

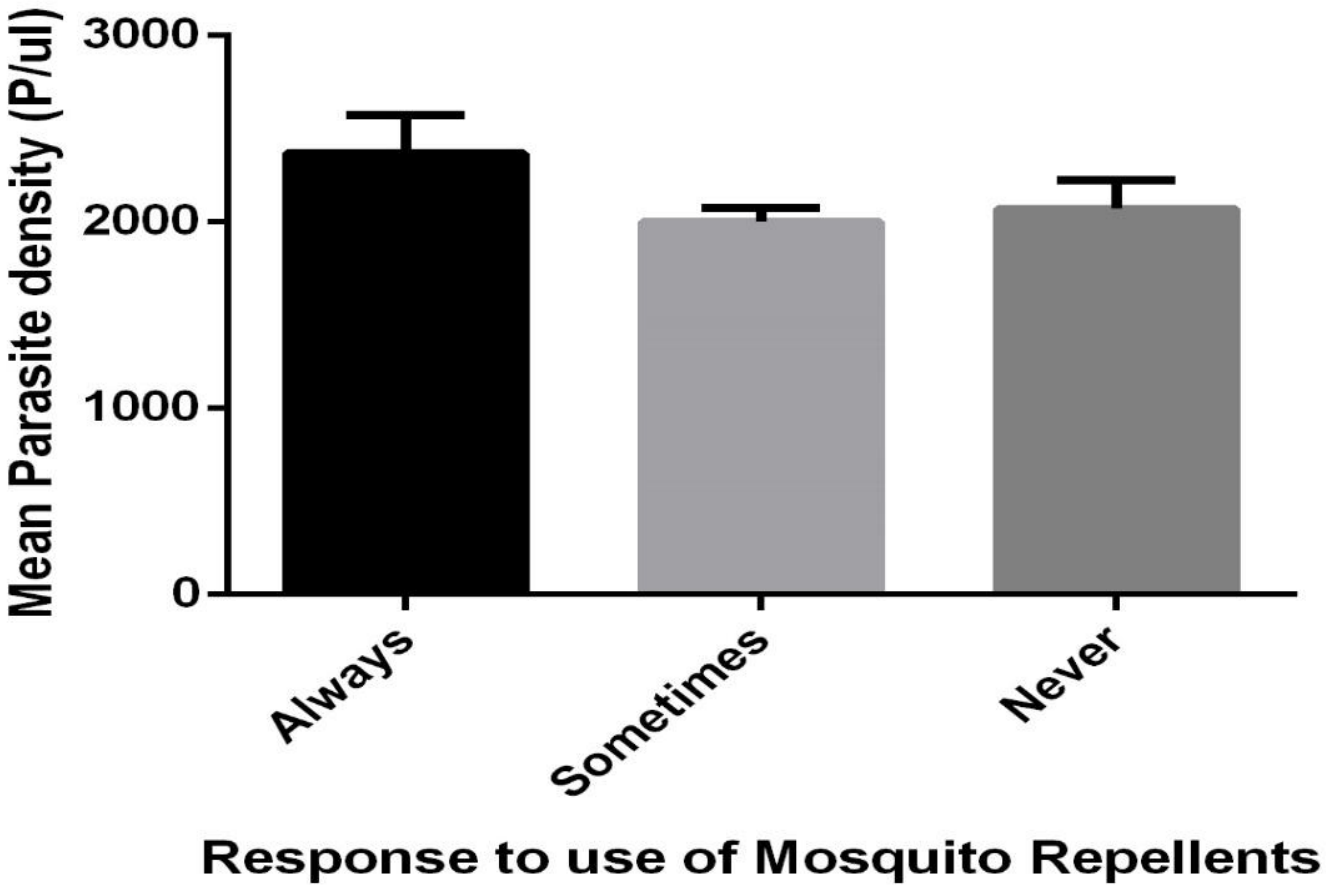



**Figure 19: Parasite Density Amongst the Children Population with respect to use of Repellent**

### 4.1.7 Use of Mosquito Net with Respect to Mothers' Highest level of Education Amongst Children Cohort.

Figure 20 detailed the frequency of mosquito net use with respect to Mothers' Highest level of education amongst the children cohort. A two way Anova was used to assess the statistical significance of the results. *P* value was set at $p \leq 0.05$. Within the response group with primary Education as the highest level of education, 37 persons said 'Always', 50 persons said 'Never' and 4 persons said that they 'Sometimes' use the mosquito net. In the response group that had secondary education as their highest level of education, 30 said 'Always' to the use of mosquito net, 72 said 'Never' to the use of mosquito net while 5 uses the net 'Sometimes'. Also, in the study group with primary education, 18 persons said 'always', 4 persons indicated using the nets 'Somtimes' while 31 persons indicated 'Never'. The two way Anova results showed that only the row factors were statistcally significant ( $p = 0.0185$) while the column factor was not significat ( $p = 0.2508$).

### 4.1.8 Distribution of *Plasmodium* species in 250 samples of Asymptomatic Children

Figure 21 detailed the different species of *Plasmodium* present in the children population. Polymerase chain reaction was used to confirm the presence of these species. The frequency table above showed that *Plasmodium falciparum* were 48 (19.2%) in number, while

*Plasmodium malaria* and *Plasmodium vivax* were 86 (34.4 %) and 16 (6.4%) respectively. Molecular analysis could not detect *Plasmodium ovale*.

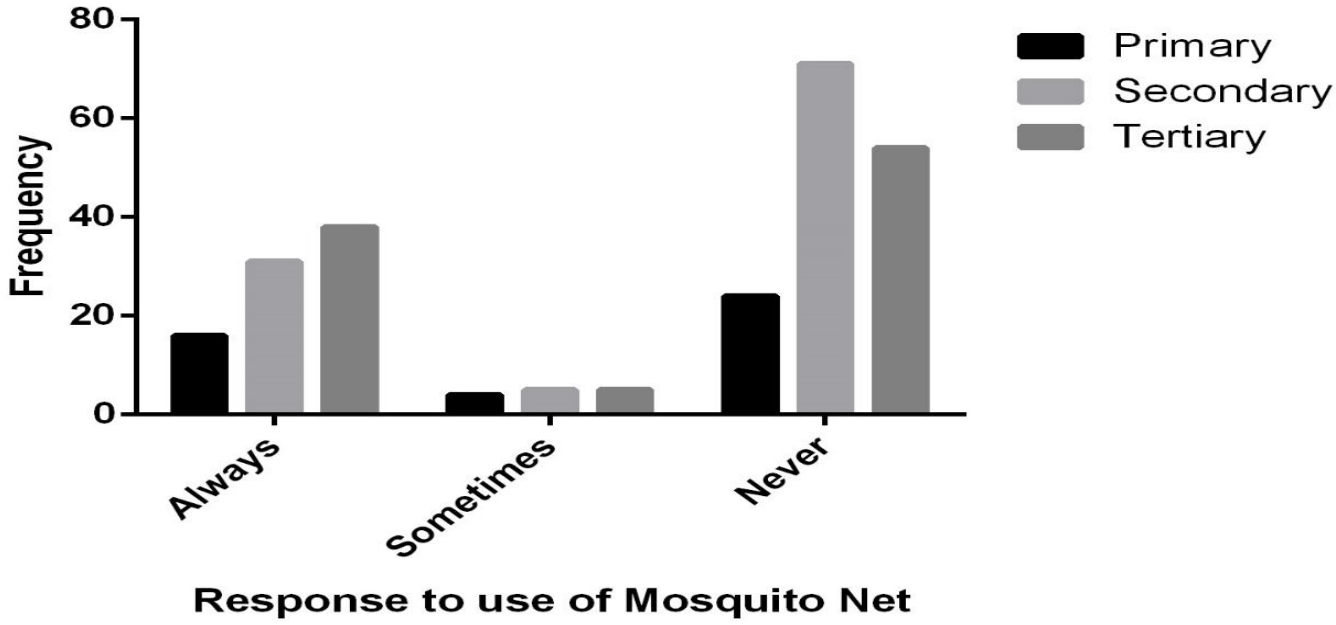
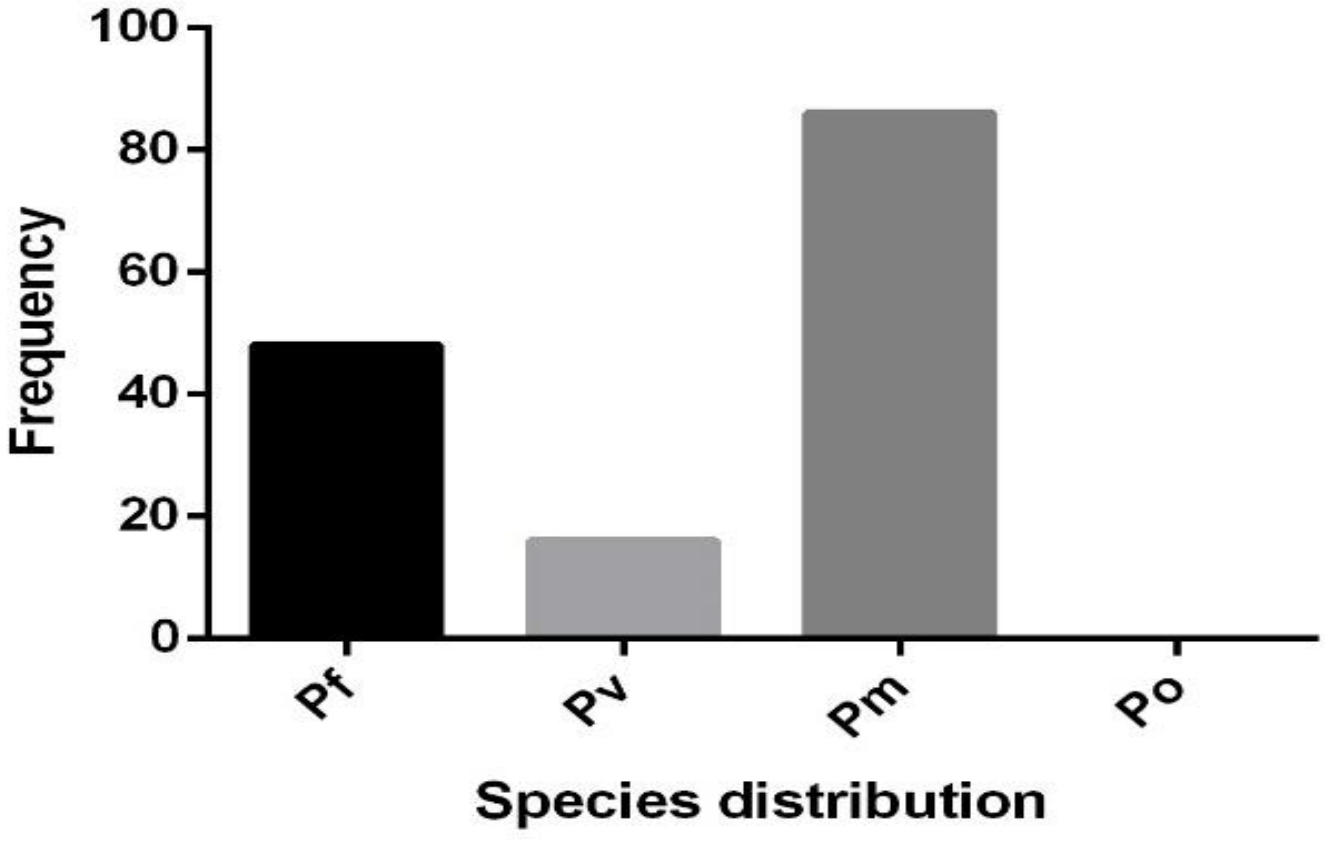



**Figure 20: Use of Mosquito Net with Respect to Mothers' Highest level of Education Amongst Children Cohort.**

**Figure 21: Distribution of *Plasmodium* species in 250 samples of Asymptomatic Children**



### 4.1.9Frequency of mixed infections observed in the Children Cohort

Figure 22 showed the various frequencies of the mixed infections observed in the children cohort. 21(17.5%) persons had *Plasmodium falciparum* mixed with *Plasmodium malaria.* 1 (0.8 %) had *Plasmodium falciparum* mixed with *Plasmodium vivax.* Mixed infections involving *Plasmodium malaria* and *Plasmodium vivax* were 4 (3.3 %), while the mixed infection involving the three species were 1 (0.8 %).

### 4.1.10Distribution of Single and Mixed infection in the Children Population

Figure 23 below showed the distribution of single and mixed infection in the children population. From the figure above, 25 (10 %) of the population had *P. falciparum* as single infection. 10 (4 %) of the population had *P.vivax* as single infection. 60 ( 24 % ) of the children population had *P. malariae* as single infection. There were presence of poly-infections amongst the population. 21 (8.4 %) had mixed infection with *P.falciparum* and *P.malariae*(PF+PM). 1 (0.4 %) had mixed infection with *P. falciparum* and *P. vivax* while mixed infections involving *P. falciparum*, *P. malarae* and *P. vivax* were 1 (0.4 %). Some of the samples were negative of all the species characterized. Of 250 samples characterized, 130 ( 52 %) were negative.

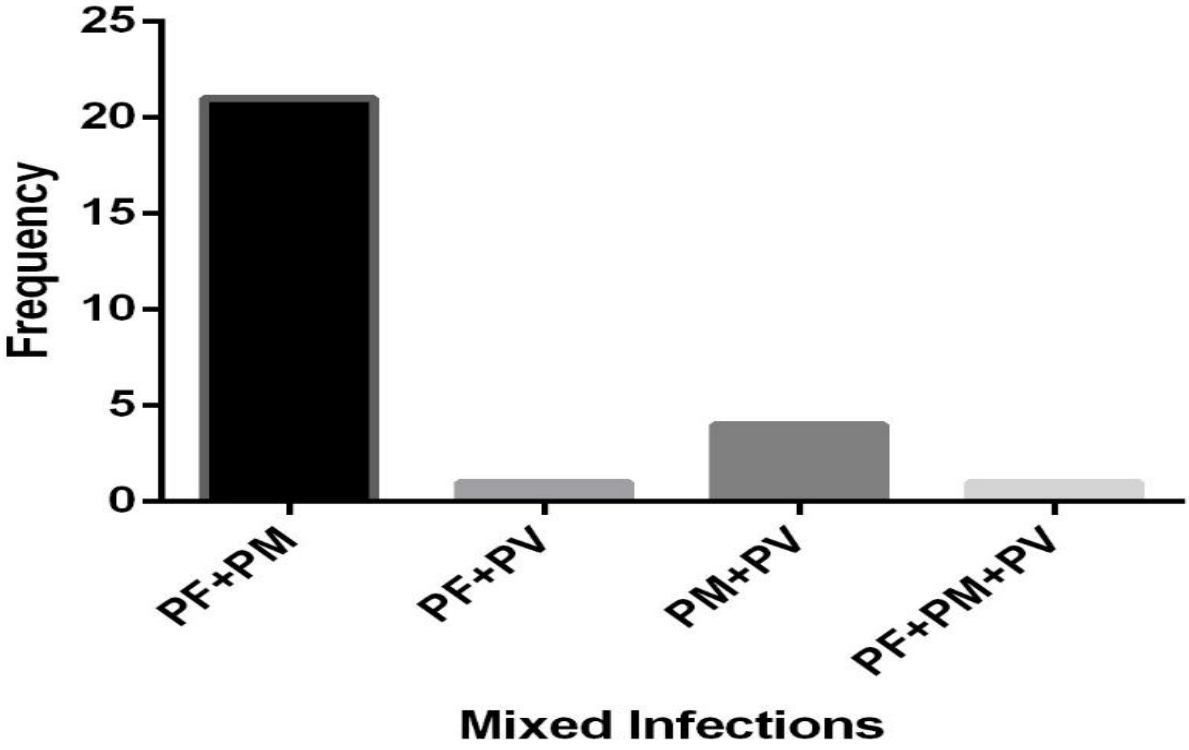



**Figure 22: Frequency of mixed infections observed in the Children Cohort**

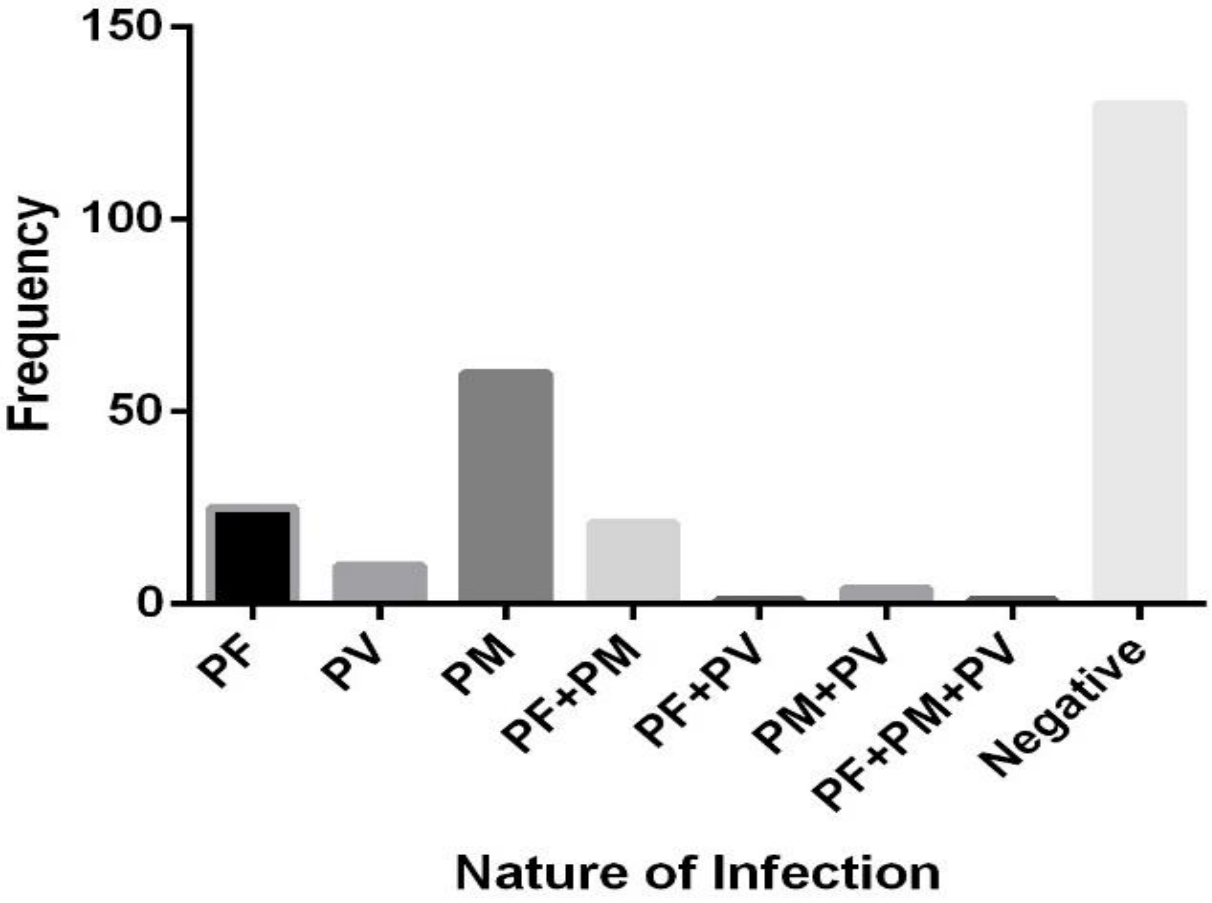



**Figure 23: Distribution of Single and Mixed infection in the Children Population**

### 4.1.11 Real Time Detection of *Plasmodium vivax* species in the Children samples

Realtime PCR was used to confirm the presence of *P.vivax* previously identified through nested PCR. This was done to clear the doubts of false positive results that sometimes arise with conventional PCR machine. Figure 24 is the amplification curve observed after the PCR runs. Total of 16 samples were subjected to real time PCR procedure. The samples were ran in duplicate with a positive control (Confirmed positive *P.vivax* from Ethiopia). A close observation of the figure above showed that the 16 samples were all positive for *P.vivax* with varied cp values.

### 4.1.12 Sequence Analysis of GATA 1 Transcription factor for Duffy Antigen

Gata 1 transcription factor was amplified using nested PCR. The PCR products were sequenced. Using the sequencer software ( Version 4.0), the sequences were analysed for the duffy antigen genotype at the promoter region. Figure 25 is the output of the sequencer after the analysis which showed that the sequences were duffy negative individual ( T→C at position -46).

### 4.1.13 Duffy Antigen Chemokine Receptor Gene Allele Variant at codon 42

Exon 2 of the DARC gene was amplified using nested PCR. The PCR products were sequenced and FYA and FYB allele variants and common polymorphisms associated withDuffy negativity were identified. Figure 26 below showed the mutation at codon 42. Here, the guanin had been replaced by adenin resulting to amino acid change from glycine (G) to aspartic acid (D) which is typical of FYB allelevariant.

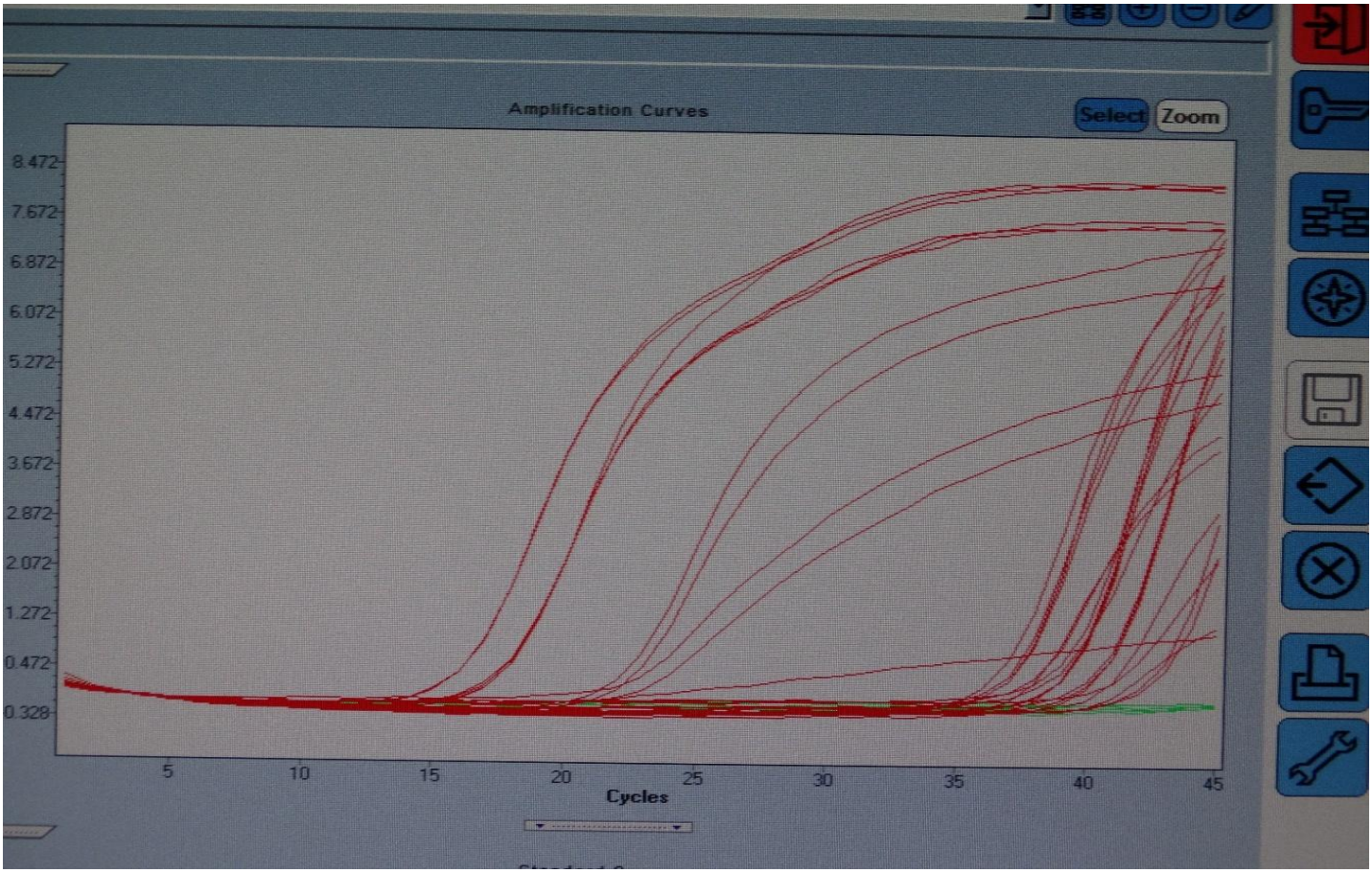



**Figure 24: Real Time Detection of *Plasmodium vivax* species in the Children samples**

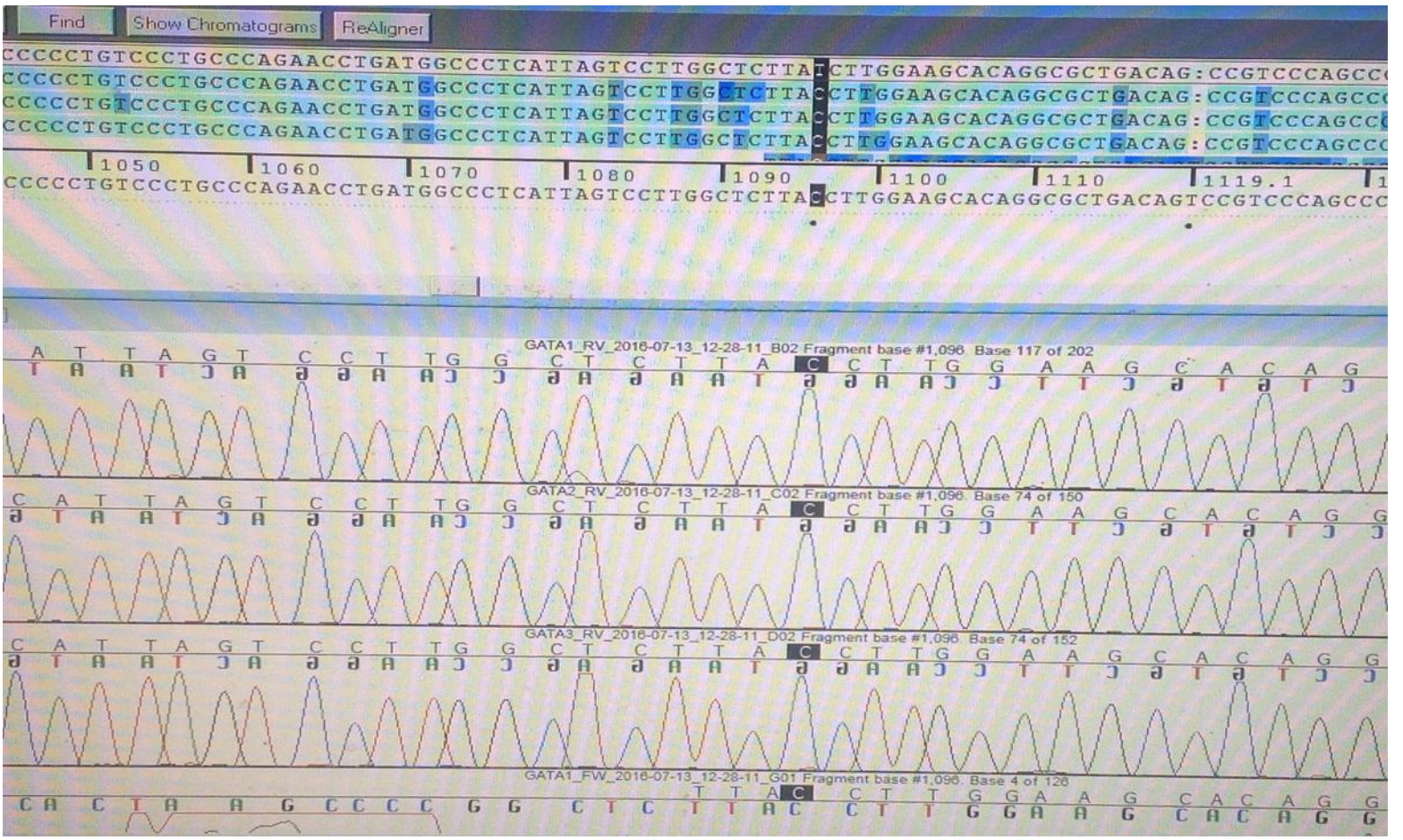



**Figure 25: Sequence Analysis of GATA 1 Transcription factor for Duffy Antigen**

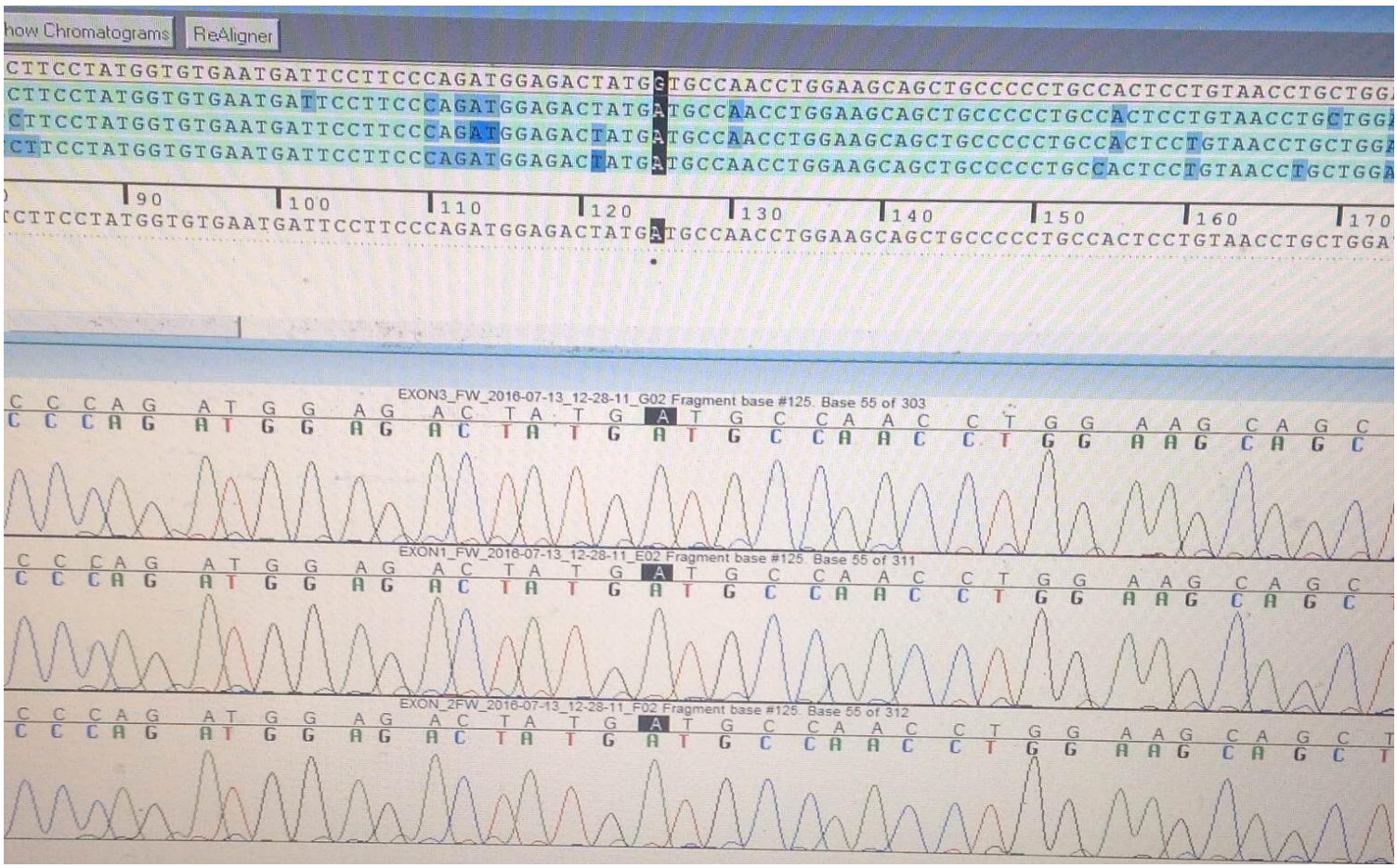



**Figure 26: Duffy Antigen Chemokine Receptor Gene Allele Variant at codon 42**

### 4.1.14Duffy Antigen Chemokine Receptor Gene Allele Variant at codon 89

Figure 27 showed the allele variant of duffy antigen at codon 89. The $Fy^{bweak}$ antigen is assessed at codon 89 (cgc encodes R, $Fy^{b}$; tgc encodes C, $Fy^{bweak}$). Here, there is no mutation at the codon. The Cytosine was not replaced by Thymine, hence we have $Fy^{b}$ variant still indicated at that codon.

### 4.1.15 Frequency (%) and number (no) of the *Pfmdr1* mutations in Children Population

The *Pfmdr1* gene was sucessfully sequenced in 36 samples amongst the *P.falciparum* found in the children samples. The results for *pfmdr1* polymorphisms are shown in Table 9. The frequency of the N86Y was 6 (16.67 % ) for the mutants and 30 (83.33 %) for the wild type. Y184F had frequency of 14 ( 38.88 % ) for the mutants and 19 (52.78 % ) for the wild type. S1034C and N1042D had 100% wild type with no mutants observed in the samples sequenced. Samples possessing heterozygous alleles were also observed in some samples. 3 (8.33 % ) of the samples had heterozygous alleles at codon 184. Also at codon 1066, 1 (2.77 % ) of the samples had heterozygote alleles. Other new mutations observed were as shown in Table 9 above excluding N86Y, Y184F, S1034C and N1042D which were already reported somewhere ( Boussaroque *et al*., 2016).

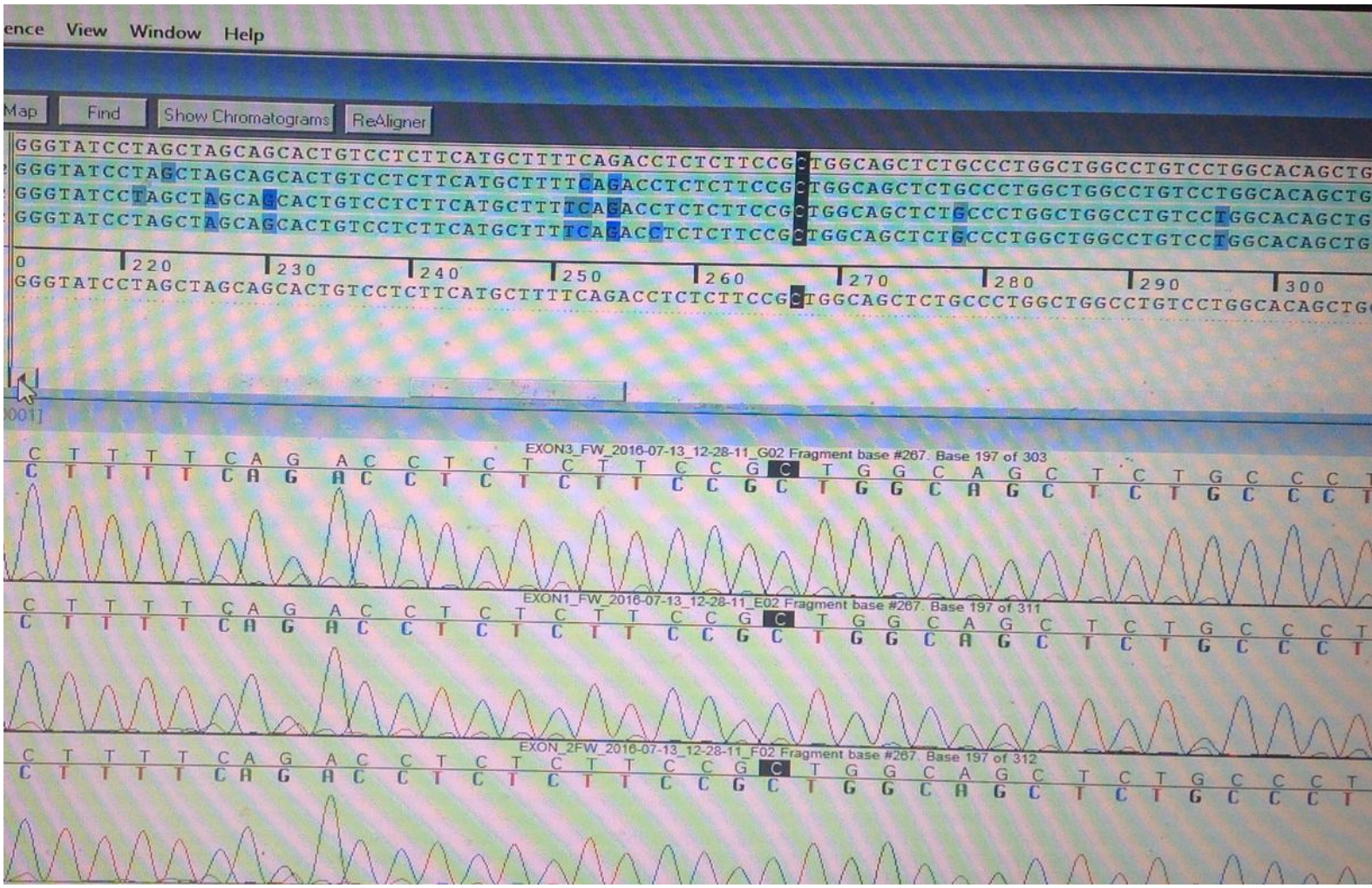



**Figure 27: Duffy Antigen Chemokine Receptor Gene Allele Variant at codon 89**

| Gene | Codon | Amino Acids Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| PfMDR1 (n=36) | 86 | N | Y | 30 (83.33) | 6 (16.67) | 0 (0) |
| | 184 | Y | F | 19 (52.78) | 14 (38.88) | 3 (8.33) |
| | 74 | F | L | 35(97.22) | 1 (2.77) | 0 (0) |
| | 164 | S | S | 35(97.22) | 1 (2.77) | 0 (0) |
| | 1066 | S | P | 35(97.22) | 0(0) | 1 (2.77) |
| | 1069 | T | T | 35(97.22) | 1 (2.77) | 0(0) |
| | 1077 | A | A | 35(97.22) | 1 (2.77) | 0(0) |
| | 1106 | N | S | 35(97.22) | 1 (2.77) | 0 (0) |
| | 1117 | I | V | 35(97.22) | 1 (2.77) | 0 (0) |

**Table 9: Frequency (%) and number (no) of the *Pfmdr1* mutations in Children Population**

| | | | | | |
|---|---|---|---|---|---|
| 1176 | R | G | 35(97.22) | 1 (2.77) | 0 (0) |
| 1122 | I | T | 35(97.22) | 1 (2.77) | 0 (0) |
| 1137 | S | S | 35(97.22) | 1 (2.77) | 0 (0) |
| 1246 | D | Y | 34(94.44) | 2(5.55) | 0(0) |
| 1034 | S | C | 36(100) | 0(0) | 0(0) |
| 1042 | N | D | 36(100) | 0(0) | 0(0) |

### 4.1.16Frequency (%) and number (n) of the *pfcrt* Mutations

Total of 23 *P.falciparum* samples were sequenced for mutations in the *Pfcrt* gene analysis. Table 10a showed the frequency of the various mutations at different codons assesed. All the samples sequenced had 100% wild type at codon 72. When assesed for mutations at codon 74, 76, and 75, it was observed that they all had 100 % mutations at the various codons. The resultant amino acid changes were all non synonymous. Three common haplotypes associated with chloroquine resistance are CVMNK, SVMNT and CVIET. When analysed for the presence of the said hyplotypes, CVIET 23 (100 %) were found (Table 10b). Non of the samples had heterozygote allele.

### 4.1.17 Frequency (%) and number (n) of Pfkelch 13 gene Mutations observed in the Children Population

23 samples were fully sequenced for assessment of single nucleotide polymorphisms (SNP) in *Pfkelch* 13 gene. Table 11a revealed codons with SNP which includes 510,515,547, 613 and 688. Apart from codon 547 that had non synonimous mutation, the rest had synonimous mutation. Frequency of mutations observed were 22 (95.65 %) for the wild type and 1 (4.34 %) for mutant. Also, Table 11b showed the outcome of the SNP anlysis on already known usual mutation in *Pfkelch* 13 gene. They all presented to be wild type across the entire samples sequenced. In general, there was no heterozygote allele observed.

| Gene | Codon | Amino Acids | | | | |
|---|---|---|---|---|---|---|
| | | Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
| Pfcrt (n=23) | 72 | C | S | 23(100) | 0(0) | 0(0) |
| | 74 | M | 1 | 0(0) | 23(100) | 0(0) |
| | 76 | K | T | 0(0) | 23(100) | 0(0) |
| | 75 | N | E | 0(0) | 23(100) | 0(0) |

**Table 10a: Frequency (%) and number (n) of the *pfcrt* Mutations**

**Table 10b: Frequency (%) and number (n) of Haplotypes observed in the *pfcrt* gene**

| Haplotypes | | |
|---|---|---|
| CVMNK n(%) | SVMNT n(%) | CVIET n(%) |
| 0(0) | 0(0) | 23(100) |

**Table 11a: Frequency (%) and number (n) of Pfkelch 13 gene Mutations observed in the Children Population**

| Gene | Codon | Amino Acids Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| Pfk13 (n=23) | 510 | V (GTG) | V (GTA) | 22(95.65) | 1(4.34) | 0(0) |
| | 515 | R (AGA) | R(AGG) | 22(95.65) | 1(4.34) | 0(0) |
| | 547 | D(GAT) | G(GGT) | 22(95.65) | 1(4.34) | 0(0) |
| | 613 | Q(CAA) | Q(CAG) | 22(95.65) | 1(4.34) | 0(0) |
| | 688 | E(GAA) | E(GAG) | 22(95.65) | 1(4.34) | 0(0) |

**Table 11b: Frequency (%) and number (n) of known mutations in Pfkelch 13 gene observed in the Children Population**

| Gene | Codon | Amino Acids Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| Pfk13 (n=23) | 476 | M | I | 23(100) | 0(0) | 0(0) |
| | 493 | Y | H | 23(100) | 0(0) | 0(0) |
| | 539 | R | T | 23(100) | 0(0) | 0(0) |
| | 543 | I | T | 23(100) | 0(0) | 0(0) |
| | 580 | C | Y | 23(100) | 0(0) | 0(0) |

### 4.1.18Frequency (%) and number (n) of the Pfatpase6 gene in the Children Population

Table 12 showed the frequency and number of mutations in the pfatpase6 gene in the Children population. Total of 17 samples were sucessfully sequenced and analysed for SNP. Mutations at codon 569 had a frequency of 2 (11.76 %) mutants and 15 (88.24 %) wild type. At codon 637, the frequency observed was 1 (5.88 %) for mutants, 15 (88.24 %) for wild – type and 1 (5.88) for heterozygote allele. Several known codons (243, 263, 431, 623 and 769) were also analysed for SNP, but they were all wild type.

**Table 12: Frequency (%) and number (n) of the Pfatpase6 gene in the Children**

| Gene | Codon | Amino Acids Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| Pfatpase6 n=17 | 569 | AAT | AAA | 15(88.24) | 2(11.76) | 0(0) |
| | 639 | GGC | GAC | 15(88.24) | 1(5.88) | 1(5.88) |
| | 243 | H | Y | 17(100) | 0(0) | 0(0) |
| | 263 | L | E | 17(100) | 0(0) | 0(0) |
| | 431 | E | K | 17(100) | 0(0) | 0(0) |
| | 623 | A | S | 17(100) | 0(0) | 0(0) |
| | 769 | S | N | 17(100) | 0(0) | 0(0) |

**Population**

## 4.2 Pregnant Women Cohort

### 4.2.1 Demographic Characteristics of the Pregnant Women Subjects

Table 13 deatiled the characteristics of the study population ( Pregnant women). The mean age of the population was 31.10 ± 0.35. The age range was from 19 years to 59 years. Sex ratio was found to be 1 because only females constitutes the population. Mean parasitaemia and parasite density range were 1897.7 ± 65.69 and 240 to 5600 respectively

### 4.2.2Mean Parasite density Amongst Pregnant women Cohort with respect to Age

Mean parasite density were correlated with age of the population using Anova, Brown – Forsythe test and Bartlett's test ($P \leq 0.05$). Figure 28 is the outcome of the analysis. The age ranges compared were 19 - 25, 26 – 35 and 36 – 59. The ranges were chosen with understanding of the development of antibodies as one advances in age. The mean parasite densities for the 19 -25, 26 -35 and 36 – 59 ranges are 2041.14 ± 202.21 P/µL, 1881.07 ± 75.81 P/µL and 1855.51 ± 136.38 P/µL respectively. The analysis also revealed that the values were not statistically significant with *P* values of 0.6981, 0.6672 and 0.4392 for Anova, Brown – Forsythe test and Barlett's test respectively.

### 4.2.3Use of Mosquito Nets Amongst Pregnant Women in Nnewi, South East, Nigeria

The use of mosquito net was assesed amongst the pregnant women cohort in Nnewi, South East Nigeria. Figure 29 above detailed the percentage use of Mosquito nets as observed in the pregnant women population. 26.64% of the population said 'Always' in response to the use of mosquito nets. 59.46 % said 'Never' in response to the use of Mosquito nets, while 13.90 % said that they do use the net 'Sometimes'.

**Table 13: Demographic Characteristics of the Pregnant Women Subjects (n=259)**

| Characteristics of Patients | Values |
|---|---|
| Mean age | 31.10 ± 0.35 |
| Age range | 19 years to 59 years |
| Sex ratio | 1 |
| Geometric mean parasitaemia | 1897.7 ± 65.69 |
| Parasite density range | 240 to 5600 |

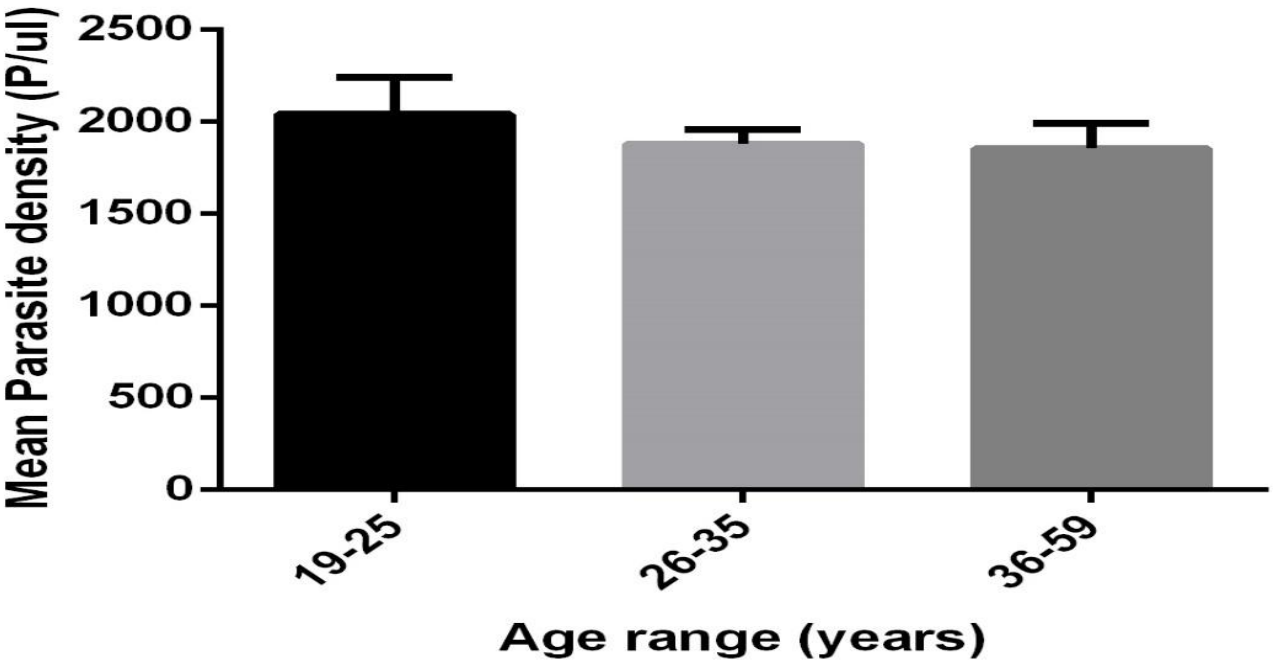



**Figure 28: Mean Parasite density Amongst Pregnant women Cohort with respect to Age**

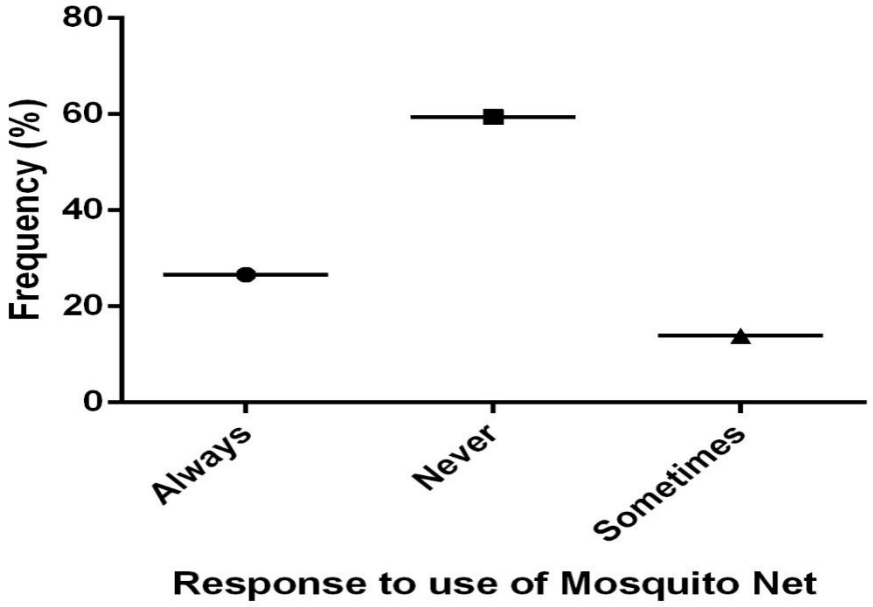



**Figure 29: Use of Mosquito Nets Amongst Pregnant Women in Nnewi, South East, Nigeria**

### 4.2.4 Mean Parasite Density in Pregnant women Population with respect to Use of Mosquito Net

Mean parasite density was correlated with the use of mosquito net as shown in figure 30 above. Anova, Brown – Forsythe and Bartlett's tests were used to compare the means of the parasite density of the various response groups in the population. P value was set at $p \leq 0.05$. The mean values of the various response groups were 1914.2 ± 123.9 (Always), 1973.3 ± 165.2 ( Sometimes) and 1872.6 ± 87.8 (Never). The analysis using the three test approches were statistically not significant ($P$ = 0.8668, 0.7736 and 0.7240 for Anova, Brown – Forthe and Bartlett's tests respectively)

### 4.2.5 Percentage Use of Mosquito Repellents Amongst Pregnant Women in Nnewi, Nigeria

Use of Mosquito repellent amongst the Pregnant women Population was assessed. Figure 31 below presented the outcome in percentage. Around fiftheen percent ( 15.44 %) of the population uses mosquito repellent. Sixty five percent (65.25%) of the population appear not to be using mosquito repellent, while 19.31 % uses it'Sometimes'.

### 4.2.6 : Mean Parasite Density of the Pregnant women Population with respect to their use of Mosquito Repellent.

Mean parasite density was compared with the various responds groups in the use of mosquito repellant (Figure 32). Anova, Brown – Forsythe and Bartlett's tests were used for the analysis. $P$ value was set at $p \leq 0.05$. It was observed that response group that said 'Always' had 1986 ± 171.6 Mean parasite density (P/µL). The response group that said 'Never'had 1842.72 ± 83.3 mean parasite density (P/µL), while the response group that said 'Sometimes' had 2012.8 ± 134.2 mean parasite density (P/µL). The analysis showed that the three tests used to compare the means of the different response groups had $p$ values greater than 0.05 ( not statistically significant). Their different $p$ value outcome were 0.5164, 0.6887 and 0.5217 for Anova, Brown – Forsythe and Bartlett's tests respectively.

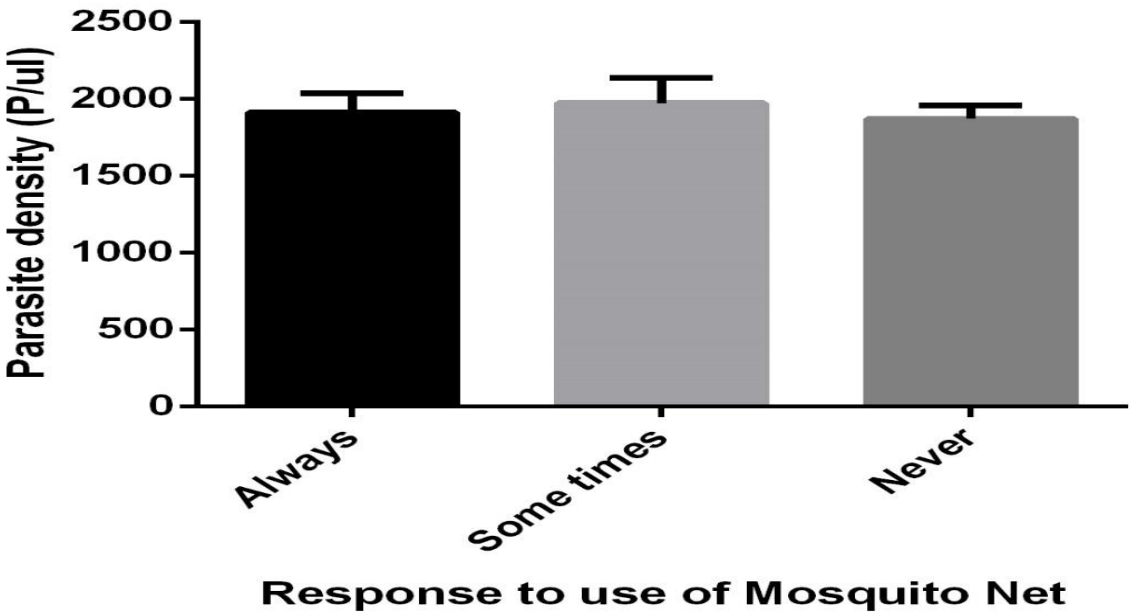



**Figure 30: Mean Parasite Density in Pregnant women Population with respect to Use of Mosquito Net**

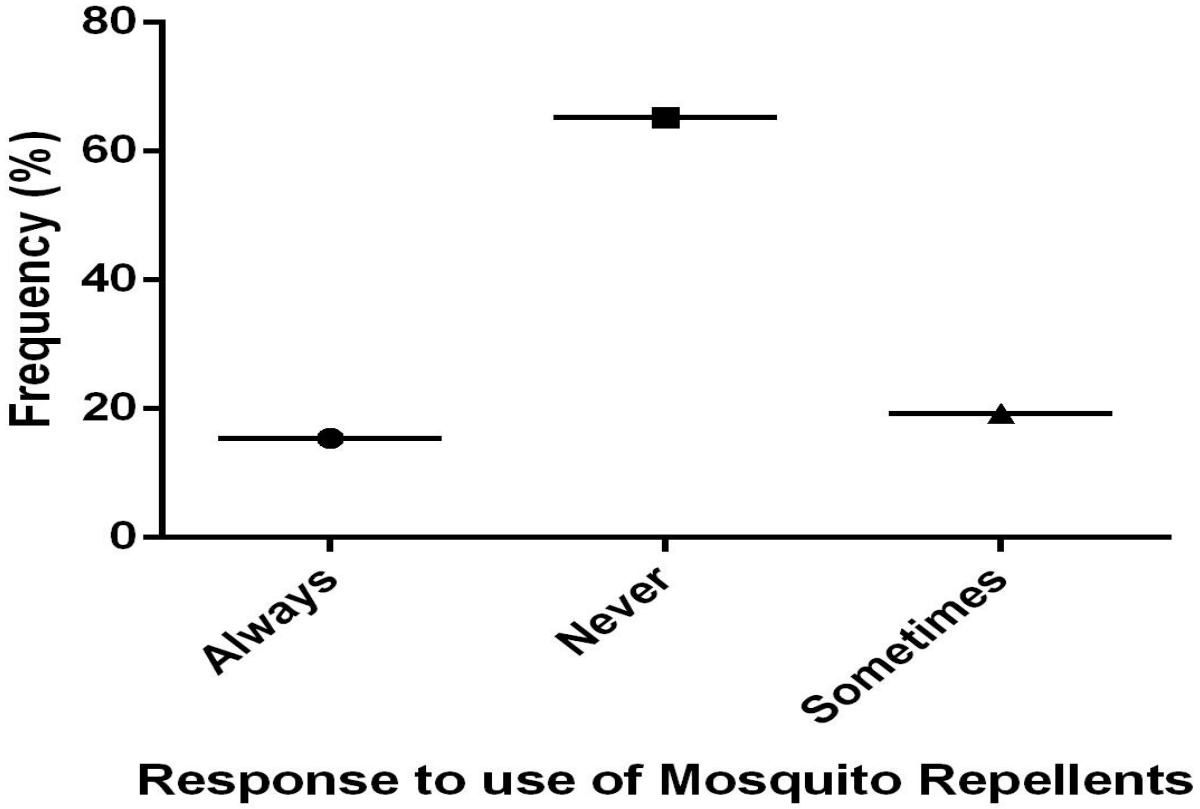



**Figure 31: Percentage Use of Mosquito Repellents Amongst Pregnant Women in Nnewi, Nigeria**

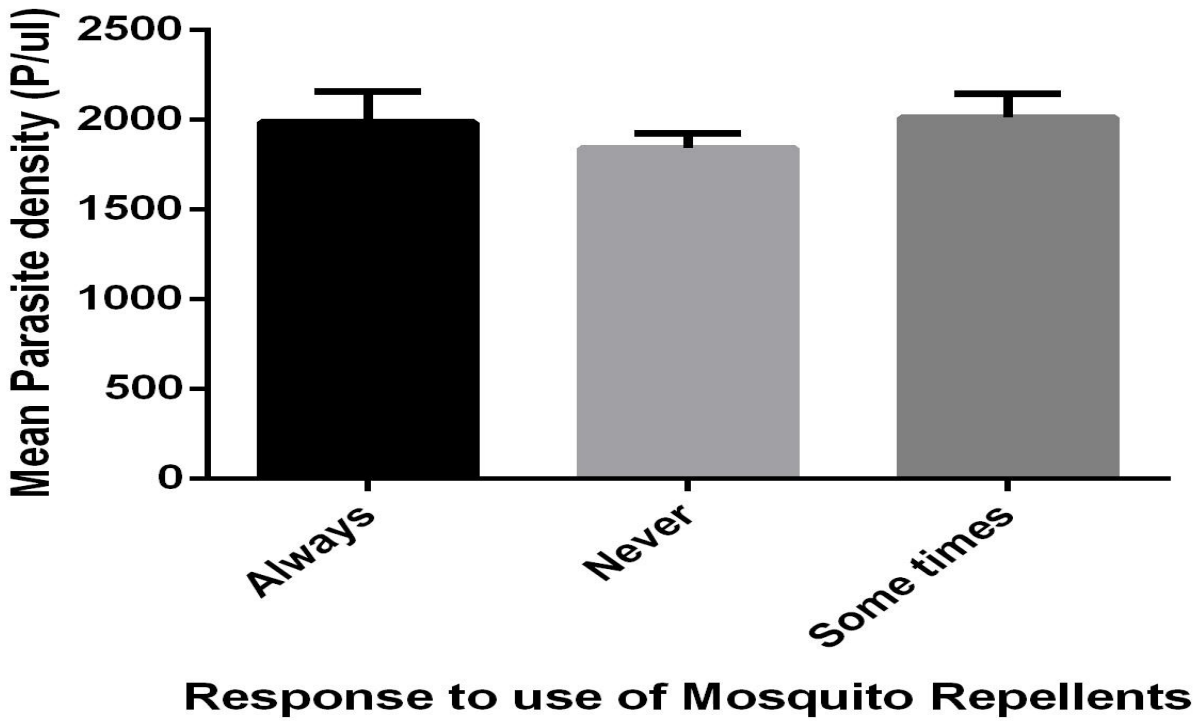



**Figure 32: Mean Parasite Density of the Pregnant women Population with respect to their use of Mosquito Repellent.**

### 4.2.7: Highest level of Education with Respect to Use of Mosquito Net Amongst the Pregnant women Cohort

Figure 33 detailed the frequency of mosquito net use with respect to Highest level of education amongst the pregnant women cohort. A two way Anova was used to assess the statistical significance of the results. *P* value was set at $p \leq 0.05$. Within the response group with primary Education as the highest level of education, 8 (3.09%) said 'Always', 11 (4.25 %) persons said 'Never' and 2 (0.77 %) said that they 'Sometimes' use the mosquito net. In the response group that had secondary education as their highest level of education, 20 (7.72 %) said 'Always' to the use of mosquito net, 68 (26.25%) said 'Never' to the use of mosquito net while 15 (5.79 %) uses the net 'Sometimes'. Also in the response group that had Tertiary education as their highest level, 41 (15.83 %) said 'Always', 75 (28.96 %) said 'Never' while 19 (7.33 %) uses the net 'Sometimes'. The two way Anova results showed that both the row factors and column factors were not statistcally significant with *p* values of 0.0685 and 0.0758 respectively.

### 4.2.8 Mean Parasite Density (P/µl) with respect to Different Trimesters amongst the Pregnant Women Cohort

Mean parasite densities (P/µl) of the pregnant women in their various trimesters were compared using Anova, Brown – Forsythe and Bartlett's tests. Figure 34 above represents the outcome of the analysis. *P* value was set at $\leq 0.05$. The mean values at different trimesters were 1558 ± 133.8, 1990 ± 118.8 and 1939 ± 92.8 for first, second and third trimesters respectively. When anlysed with the above mentioned tests, the outcome was not significant statiscally. The *p* values were 0.0815, 0.1452 and 0.1478 respectively for Anova, Brown – Forsythe and Barlett's tests.

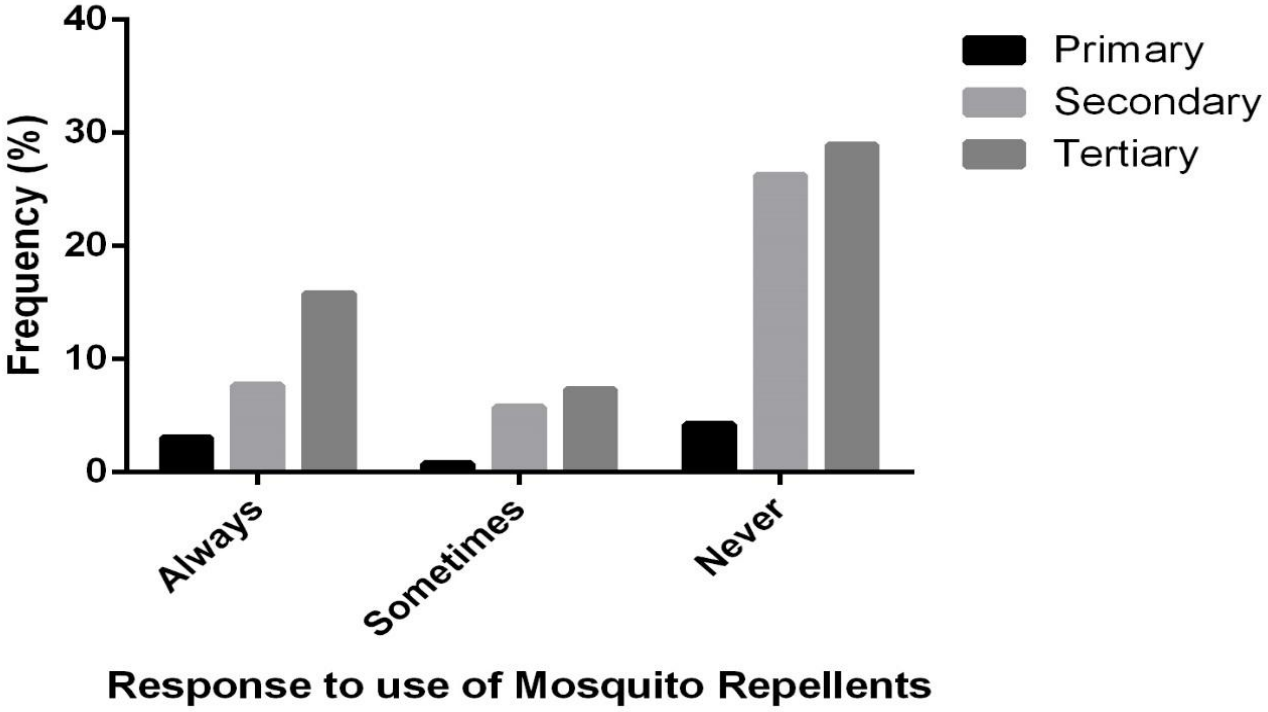



**Figure 33: Highest level of Education with Respect to Use of Mosquito Net Amongst the Pregnant women Cohort**

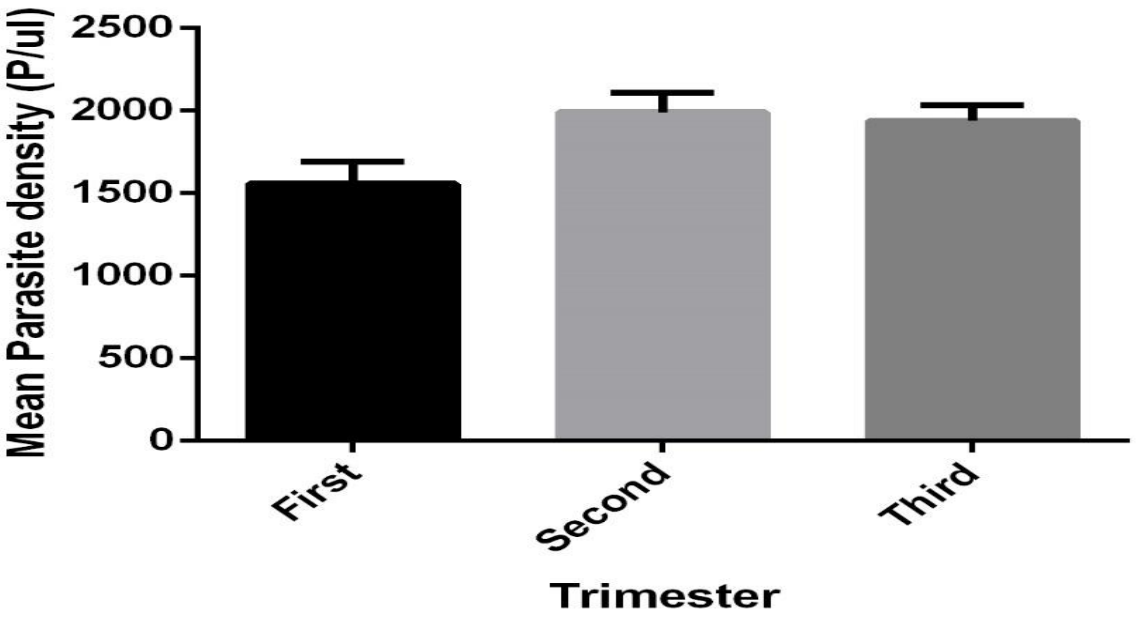



**Figure 34: Mean Parasite Density (P/µl) with respect to Different Trimesters amongst the Pregnant Women Cohort**

### 4.2.9 : Percentage Distribution of *Plasmodium* species amongst the Pregnant Women Cohort

Nested PCR with appropriate primers were used to molecularly assess for the various species of *Plasmodium* in the blood samples (n=250) of the pregnant women attending antenatal in two women hospitals at Nnewi. Of the four species assessed, only *Plasmodium falciparum* were found present { 20 (8 %)} as shown in Figure 35 below. Such was expected as many were under IPT (Intermitent Prevention Therapy).

### 4.2.10: Frequency (%) and number (no) of the *Pfmdr1* mutations in Pregnant women Population

The *Pfmdr1* gene was sucessfully sequenced in the entire 20 samples that had *P.falciparum* in their blood. The results for *pfmdr1* polymorphisms are shown in Table 14. The frequency of the N86Y was 1 (5 % ) for the mutants and 19 (95 %) for the wild type. Y184F had frequency of 4 ( 20 % ) for the mutants and 16 (80 % ) for the wild type. S1034C and N1042D had 100% wild type with no mutants observed in the samples sequenced. 1 (5 %) Heterozygous alleles was observed in codon 1090. Synonymous mutations were observed in codons 1154 and 1224. Other new mutations observed were as shown in Table 14.

### 4.2.11 : Frequency (%) and number (n) of the *pfcrt* Mutations and Possible Haplotypes of the *Plasmodium falciparum* from the Pregnant Women Cohort

Total of 11*P.falciparum* samples were sequenced for mutations in the *Pfcrt* gene analysis. Table 15 showed the frequency of the various mutations at different codons assesed and their consequent haplotypes. All the samples sequenced had 100% wild type at codon 72. When assesed for mutations at codon 74, 76, and 75, it was observed that they all had 90.90 % for mutants and 9.09 % wild type. The resultant amino acid changes were all non synonimous. Three common haplotypes associated with chloroquine resistance are CVMNK, SVMNT and

CVIET. When analysed for the presence of the said hyplotypes, CVIET 10 (90.90 %) and CVMNK 1 (9.09 %) were found (Table 15). Non of the samples had heterozygote allele.

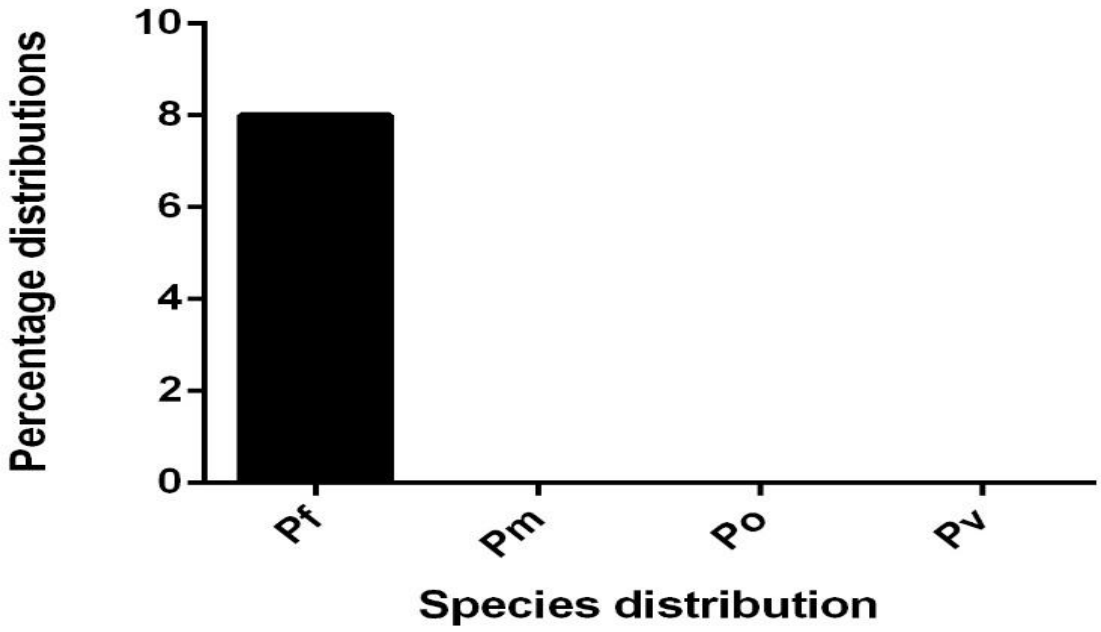



**Figure 35: Percentage Distribution of *Plasmodium* species amongst the Pregnant Women Cohort**

**Table 14: Frequency (%) and number (no) of the *Pfmdr1* mutations in Pregnant women Population**

| Gene | Codon | Amino Acids Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| PfMDR1 (n=20) | 86 | N | Y | 19(95) | 1(5) | 0(0) |
| | 158 | Y | H | 19(95) | 1(5) | 0(0) |
| | 184 | Y | F | 16(80) | 4(20) | 0(0) |
| | 1034 | S | C | 20(0) | 0(0) | 0(0) |
| | 1042 | N | D | 20(0) | 0(0) | 0(0) |
| | 1104 | K | E | 19(95) | 1(5) | 0(0) |
| | 1244 | R | G | 19(95) | 1(5) | 0(0) |
| | 1224 | T | T | 19(95) | 1(5) | 0(0) |
| | 1154 | K | K | 19(95) | 1(5) | 0(0) |
| | 1129 | K | R | 19(95) | 1(5) | 0(0) |
| | 1090 | A | A | 19(95) | 0(0) | 1(5) |
| | 1004 | Y | H | 19(95) | 1(5) | 0(0) |
| | 1246 | D | Y | 19(95) | 1(5) | 0(0) |

**Table 15: Frequency (%) and number (n) of the *pfcrt* Mutations and Possible Haplotypes of the *Plasmodium falciparum* from the Pregnant Women Cohort**

| Gene | Codon | Amino Acids Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| Pfcrt (n=11) | 72 | C | S | 11 (100) | 0(0) | 0(0) |
| | 74 | M | I | 1(9.09) | 10(90.90) | 0(0) |
| | 75 | N | E | 1(9.09) | 10(90.90) | 0(0) |
| | 76 | K | T | 1(9.09) | 10(90.90) | 0(0) |
| HAPLOTYPES | | | | | | |
| CVMNK n(%) | | SVMNT n(%) | | CVIET n(%) | | |
| 1(9.09) | | 0(0) | | 10(90.90) | | |

### 4.2.12: Frequency (%) and number (no) of the DHFR and DHPS gene mutations in Pregnant women Population

Table 16 showed the outcome of SNP analysis on DHPS and DHFR genes from *P. falciparum* from Pregnant women cohort. Total of 7 and 10 samples were fully sequenced ( those with good sequences) for dhfr and dhps respectivefully. For DHFR gene, mutations were observed at codons 511, 59 and 108, while in DHPS gene, mutations were observed at codons 431, 436, 581 and 613. While all the mutations observed were non – synonimous, heterozygous allele was observed at DHPS gene at codon 581 ( 20 %). The frequency and number of the various mutations were well shown in Table 16.

**Table 16: Frequency (%) and number (no) of the DHFR and DHPS gene mutations in Pregnant women Population**

| Gene | Codon | Amino Acids Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| DHFR (n=7) | 511 | N | I | 3(42.86) | 4(57.14) | 0(0) |
| | 59 | C | R | 2(28.57) | 5(71.43) | 0(0) |
| | 108 | S | N | 2(28.57) | 5(71.43) | 0(0) |
| DHPS(n=10) | | | | | | |
| | 431 | I | V | 6(60) | 4(40) | 0(0) |
| | 436 | S | A | 5(50) | 5(50) | 0(0) |
| | 581 | A | G | 6(60) | 2(20) | 2(20) |
| | 613 | A | S | 5(50) | 5(50) | 0(0) |

## 4.3 Adult Population Cohorts

### 4.3.1 Demographic Characteristics of the Adult Population Subjects

Table 17 deatiled the characteristics of the study population ( Adult Population). The mean age of the population was 36.83 ± 0.8537. The age range was from 11 years to 82 years. Sex ratio was found to be 0.98 (155:157). Mean parasitaemia and parasite density range were 1228.21 ± 42.21 and 160 to 4080 respectively.

### 4.3.2 Use of Mosquito Nets in an Adult Population, Nnewi South East Nigeria

The use of mosquito net was assesed in an adult population cohort in Nnewi, South East Nigeria. Figure 36 below detailed the percentage use of Mosquito nets as observed in the adult population. About 18.84 % of the population said 'Always' in response to the use of mosquito nets, 75.72 % said Never in response to the use of Mosquito nets, while 5.43 % said that they do use the net 'Sometimes'.

### 4.3.3 Mean Parasite Density with Respect to Use of Mosquito Nets in an Adult Population, Nnewi, South East Nigeria.

Mean parasite density was correlated with the use of mosquito net as shown in figure 37 below. Anova, Brown – Forsythe and Bartlett's tests were used to compare the means of the parasite density of the various response groups in the population. *P* value was set at $p \leq 0.05$. The mean values of the various response groups were 1158.67 ± 83.79 (Always), 1252.99 ± 50.11 (Never) and 1137.78 ± 185.49 ( Sometimes). The analysis using the three test approches were not statistically significant for Anova test (p = 0.3436), Brown – Forsythe (p = 0.2134) and Bartlett's tests (0.2853).

**Table 17: Demographic Characteristics of the Adult Population Subjects (n = 312 )**

| Characteristics of patients | Values |
|---|---|
| Mean age | 36.83 ± 0.8537 |
| Age range | 11 years to 82 years |
| Sex ratio (M:F) | 0.98 (155: 157) |
| Geometric mean parasitaemia | 1228.21 ± 42.21 |
| Parasite density range | 160 to 4080 |

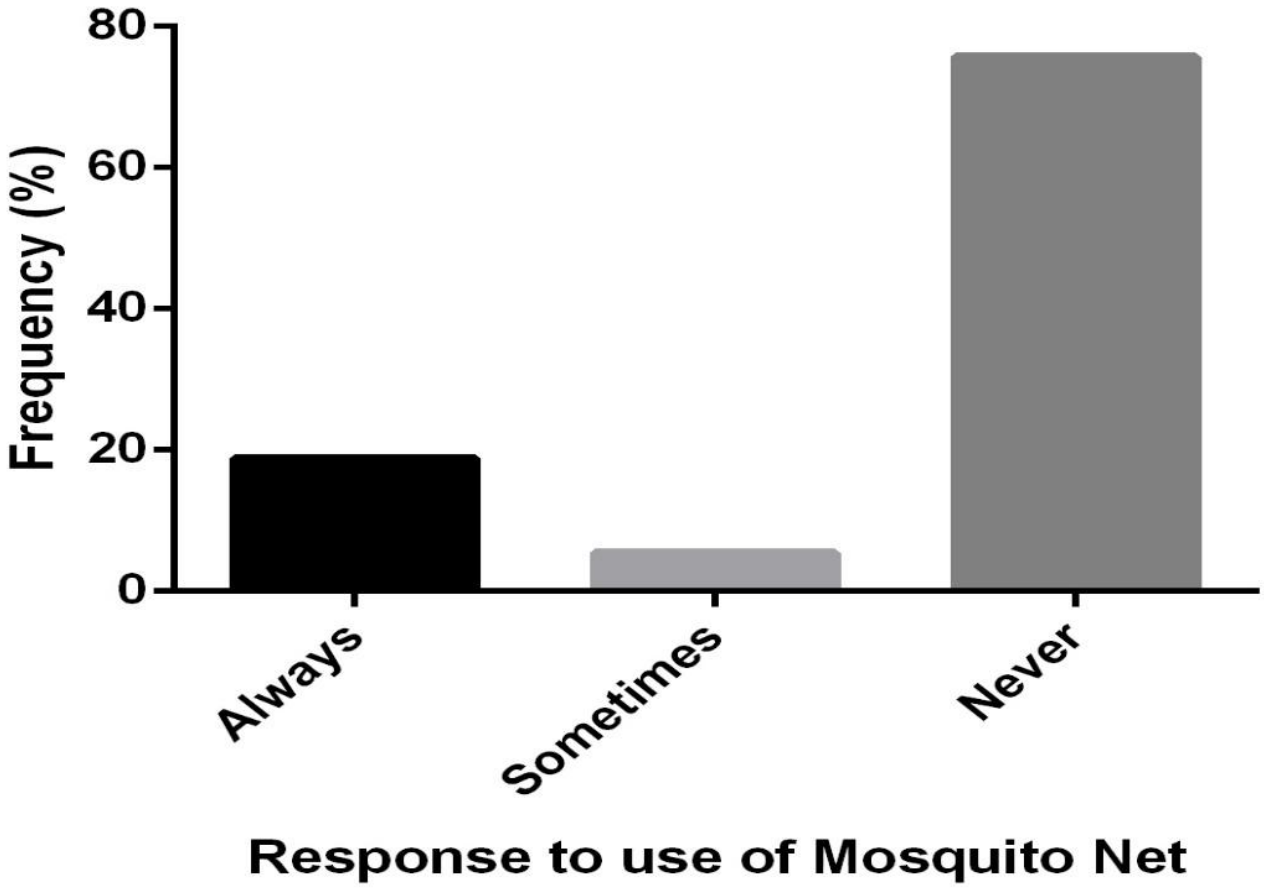



**Figure 36: Use of Mosquito Nets in an Mixed Adult Population, Nnewi South East Nigeria**

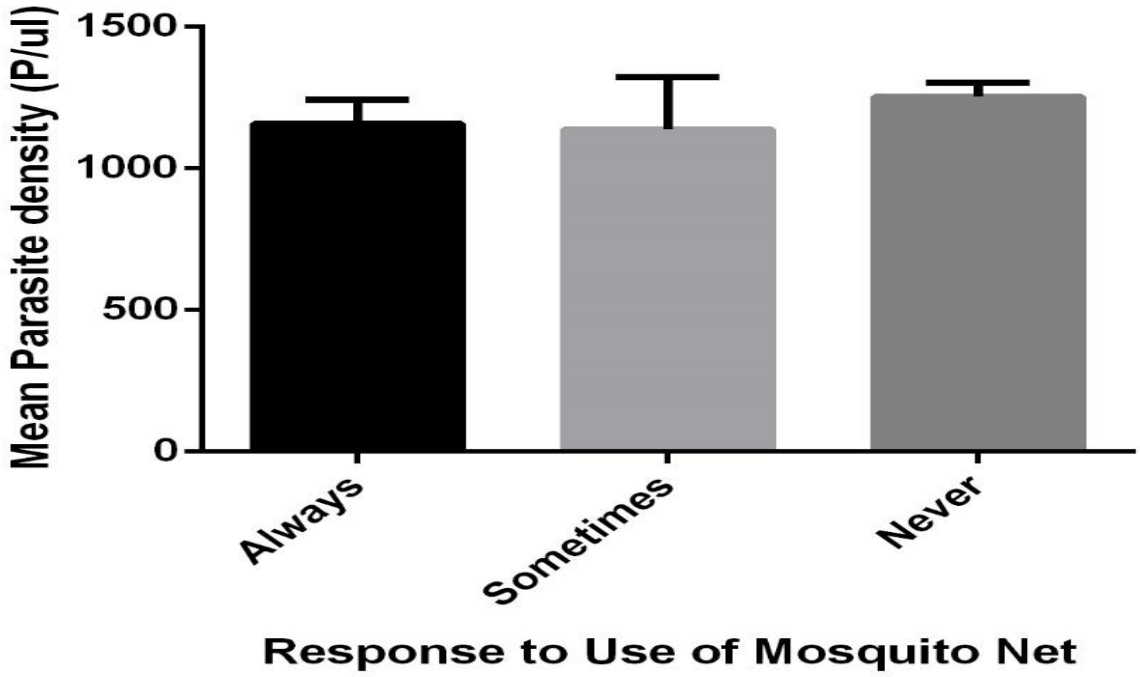



**Figure 37: Mean Parasite Density with Respect to Use of Mosquito Nets in an Adult Population, Nnewi, South East Nigeria.**

### 4.3.4: Use of Repellents in an Adult Population in Nnewi, South East Nigeria

Use of Mosquito repellent amongst the adult Population was assessed. Figure 38 presented the outcome in percentage. Around 13.42 % of the population uses mosquito repellent. About 47.60 % of the population appear not to be using mosquito repellent, while 38.98 % uses mosquito repellents sometimes.

### 4.3.5: Mean Parasite Density of an adult population Cohort with respect to their Responses to Use of Mosquito Repellents

Mean parasite density was compared with the various responds groups in the use of mosquito repellent (Figure 39) in an adult population at Nnewi, South East Nigeria. Anova, Brown – Forsythe and Bartlett's tests were used for the analysis. P value was set at $p \leq 0.05$. It was observed that response group that said 'Always' had 1100.488 ± 87.71 mean parasite density (P/µL). The response group that said 'Never' had 1288.477 ± 63.00 mean parasite density (P/µL), while the response group that said 'Sometimes' had 1196 ± 69.50 mean parasite density (P/µL). The analysis showed that the three tests used to compare the means of the diferent response groups had $p$ values greater than 0.05 ( for anova, $p = 0.3000$).

### 4.3.6 : Mean Parasite Density with Respect to Ages of those in the Mixed Adult Population at Nnewi, Nigeria

Mean parasite density were correlated with age of the population using Anova, Brown – Forsythe test and Bartlett's test ($P \leq 0.05$) . The age ranges compared were 10 – 25, 26 – 40, 41– 55 and 56 - 83. The range was from lowest to highest. The mean parasite densities for the 10 – 25, 26 – 40, 41 – 55 and 56 - 83 ranges are 1481.11 ± 98.441 P/µL, 1174.468 ± 58.66 P/µL, 1052.075 ± 87.44P/µL and 1200 ± 115.52 P/µLrespectively (Figure 40). The analysis also revealed that the values were statistically significant ( anova $p = 0.0387$).

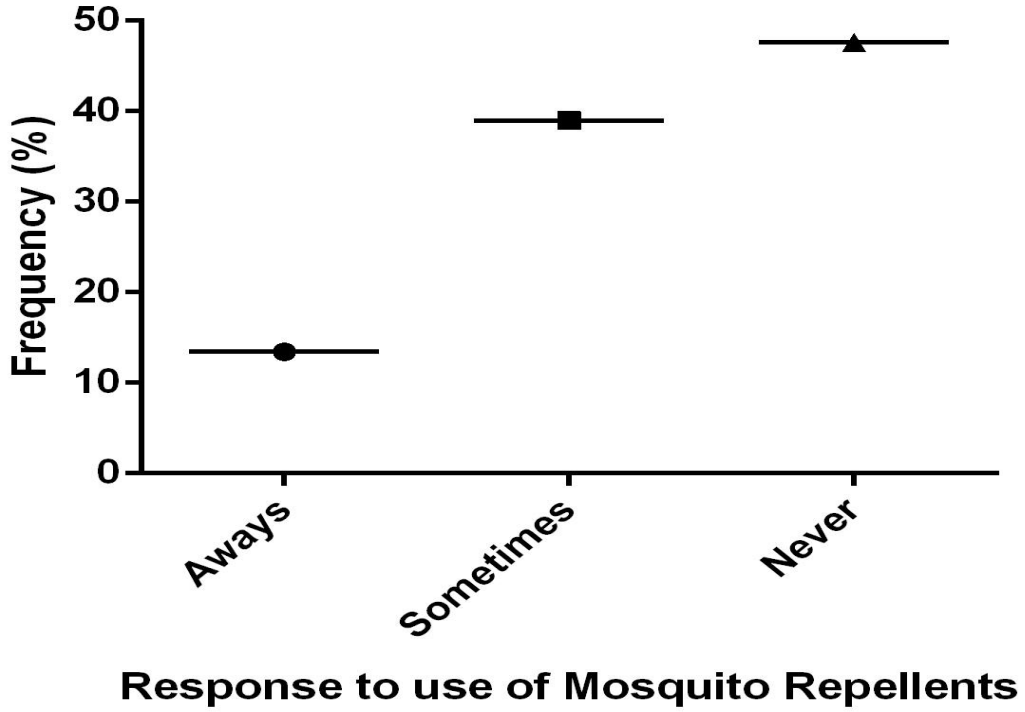



**Figure 38: Use of Repellents in an Adult Population in Nnewi, South East Nigeria**

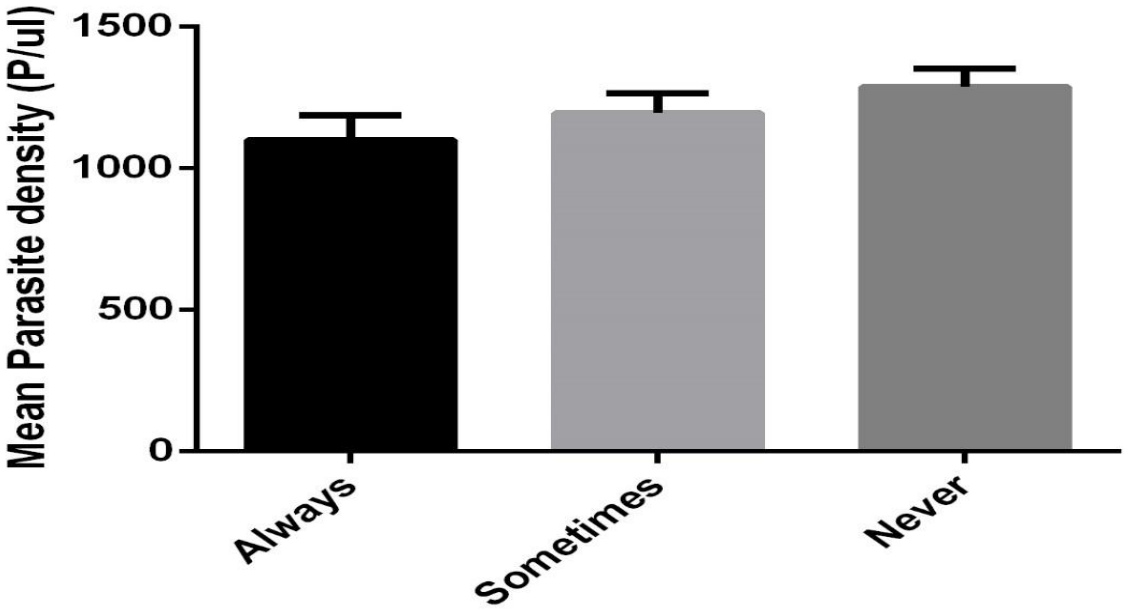



**Figure 39: Mean Parasite Density of an adult population Cohort with respect to their Responses to Use of Mosquito Repellents**

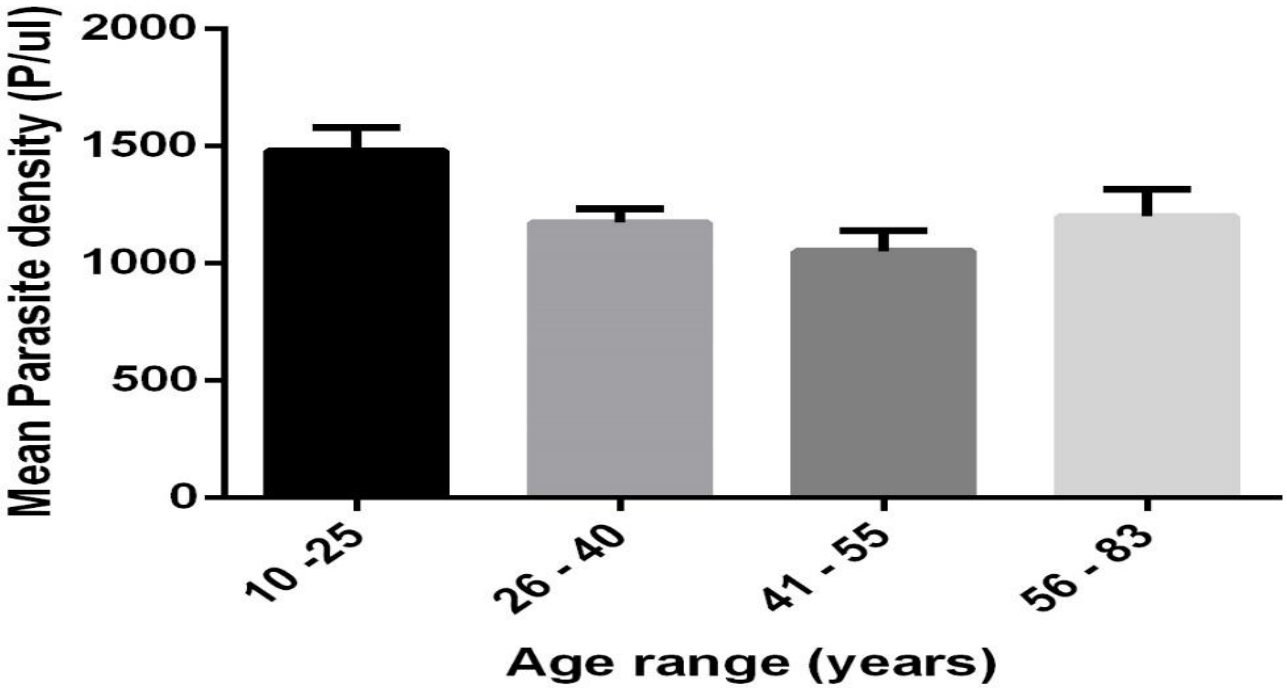



**Figure 40: Mean Parasite Density with Respect to Ages of those in the Adult Population at Nnewi, Nigeria**

### 4.3.7 :Mean Parasite Density with respect to Sex in an Adult Population in Nnewi, South East Nigeria

The mean parasite density of male and female in the adult population were compared using unpaired T test ($p \leq 0.05$). Figure 41 below is the outcome of the analysis. Female had mean value of 1206 ± 58.95 while the male had 1205 ± 59.81, although they were not statiscally significant.

### 4.3.8 : Response to Use of Mosquito Net with Respect to their Respective Highest level of Education Amongst the Adult Population

Figure 42 detailed the frequency of mosquito net use with respect to Highest level of education amongst the adult population cohort. A two way Anova was used to assess the statistical significance of the results. *P* value was set at $p \leq 0.05$. Within the response group with primary Education as the highest level of education, 2.88% said 'Always', 14.38% said 'Never' and 0.32% persons said that they 'Sometimes' use the mosquito net. In the response group that had secondary education as their highest level of education, 6.07 % said 'Always' to the use of mosquito net, 36.10 % said 'Never' to the use of mosquito net while 3.19 % uses the net 'Sometimes'. The two way Anova results showed that only the row factors were statistcally significant ( $p = 0.0149$) while the column factor was not significat ( $p = 0.2379$).

### 4.3.9 : Frequency of Single Infections Across the Adult Population Cohort

Nested PCR with appropriate primers were used to molecularly define the various species of *Plasmodium* present in the samples analysed. Two hundred and twenty five (225) samples were subjected to molecular identification. Figure 43 showed the Frequency of samples with

single infection with *Plasmodium* species. The distribution of the mono infection were 19 for *Plasmodium falciparum* , 66 for *Plasmodium malariae*, 7 for *Plasmodium vivax*.

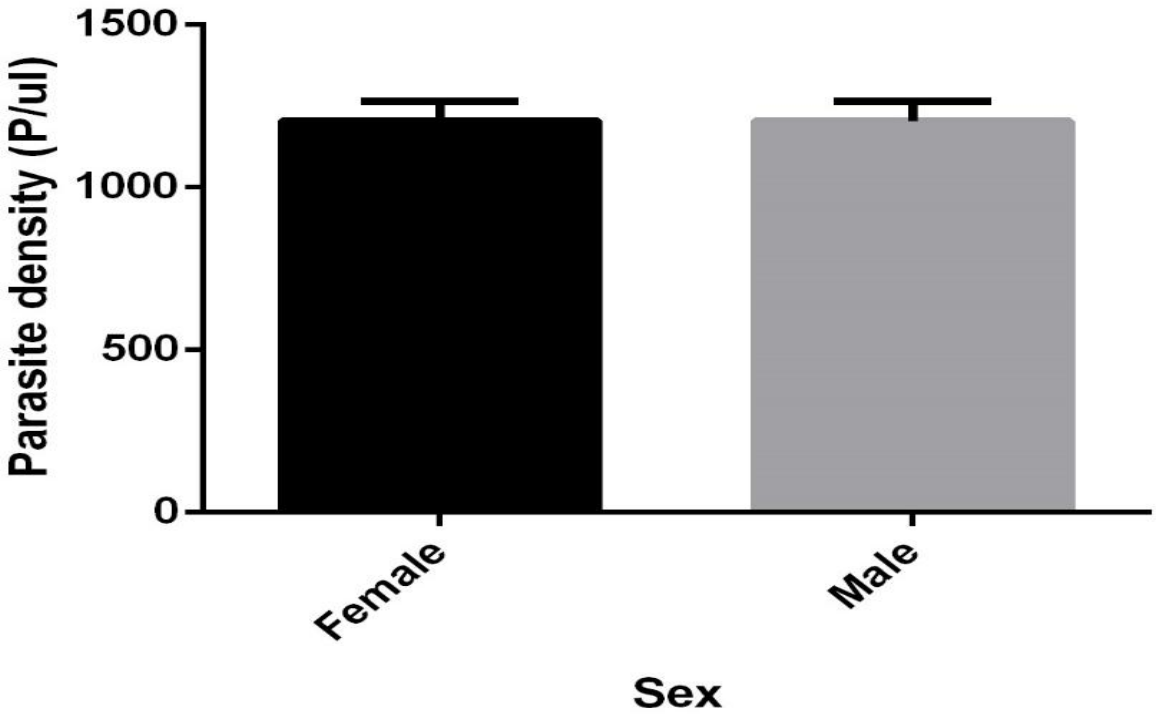



**Figure 41: Mean Parasite Density with respect to Sex in the Mixed Adult Population in Nnewi, South East Nigeria**

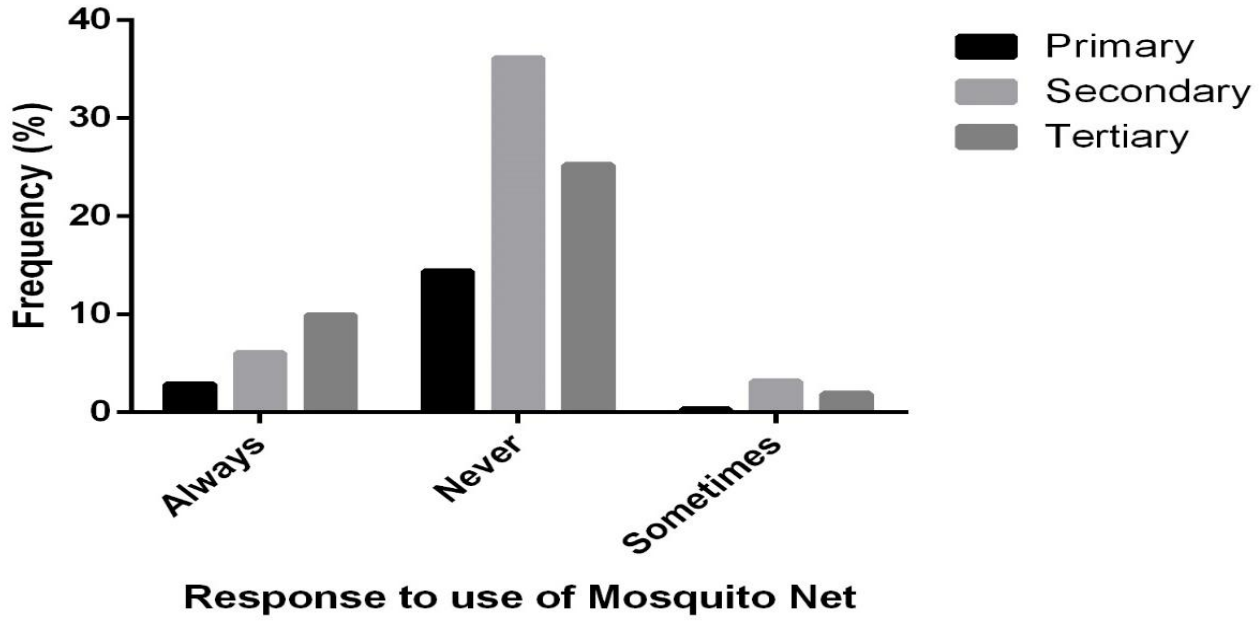



**Figure 42: Response to Use of Mosquito Net with Respect to their Respective Highest level of Education Amongst the Adult Population**

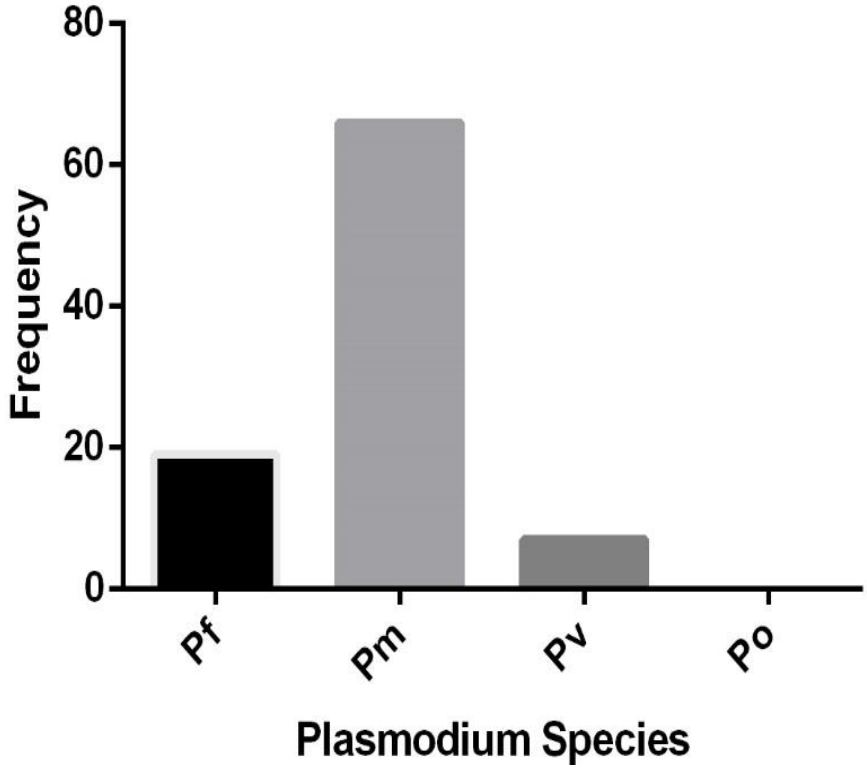



**Figure 43: Frequency of Single Infections Across the Adult Population Cohort**

### 4.3.10 : Frequency of Mixed infection Amongst the Adult Population Cohort

Figure 44 showed the various frequencies of the mixed infections observed in the adult population cohort . Thirteen [13 (5.2%) ] persons had *Plasmodium falciparum* mixed with *Plasmodium malaria.* Around3(1.2 %) had *Plasmodium falciparum* mixed with *Plasmodium vivax*while mixed infections involving *Plasmodium malaria* and *Plasmodium vivax* were 4 (3.3 %).

### 4.3.11: *Plasmodium* species distribution in the Adult Population Cohort

Figure 45 detailed the different species of *Plasmodium* present in the adult population cohort. Polymerase chain reaction was used to confirm the presence of these species. Figure 35 above showed that *Plasmodium falciparum* were 35 (15.56%), while *Plasmodium malaria* and *Plasmodium vivax* were 84 (37.33 %) and 15 (6.67 %) respectively. Samples that were negative were 91 (40.44 %). Molecular analysis could not detect *Plasmodium ovale*.

### 4.3.12: Frequency (%) and number (no) of the *Pfmdr1* mutations in the Adult Population Cohort

The *Pfmdr1* gene was sucessfully sequenced in 26 samples amongst *the P.falciparum* found in the adult population samples. The results for *pfmdr1* polymorphisms were shown in Table 18. The frequency of the N86Y was 0 (0 % ) for the mutants and 26 (100 %) for the wild type. Y184F had frequency of 6 ( 23.08 % ) for the mutants and 20 (76.92 % ) for the wild type. Heterozygous alleles were found at codons 1114 (3.85%) and 1143 (3.85%). Synonymous mutation was observed at codon 1157 (3.85%). Other mutations observed were as shownin Table 18.

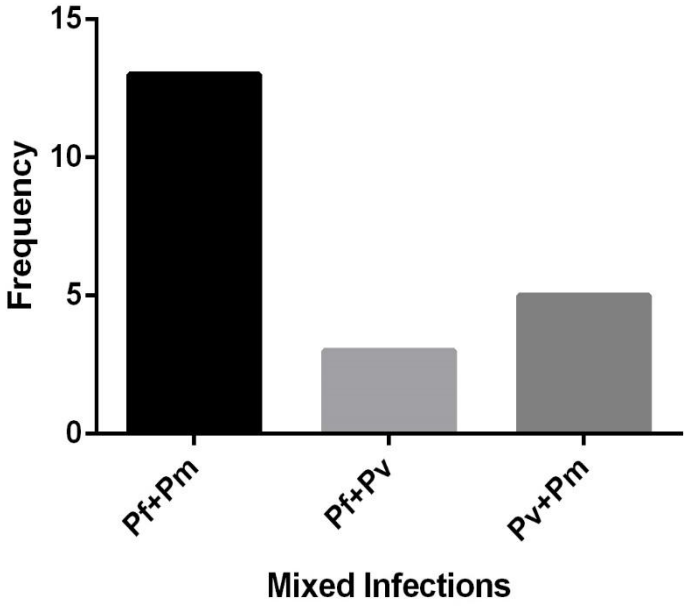



**Figure 44: Frequency of Mixed infection Amongst the Adult Population Cohort**

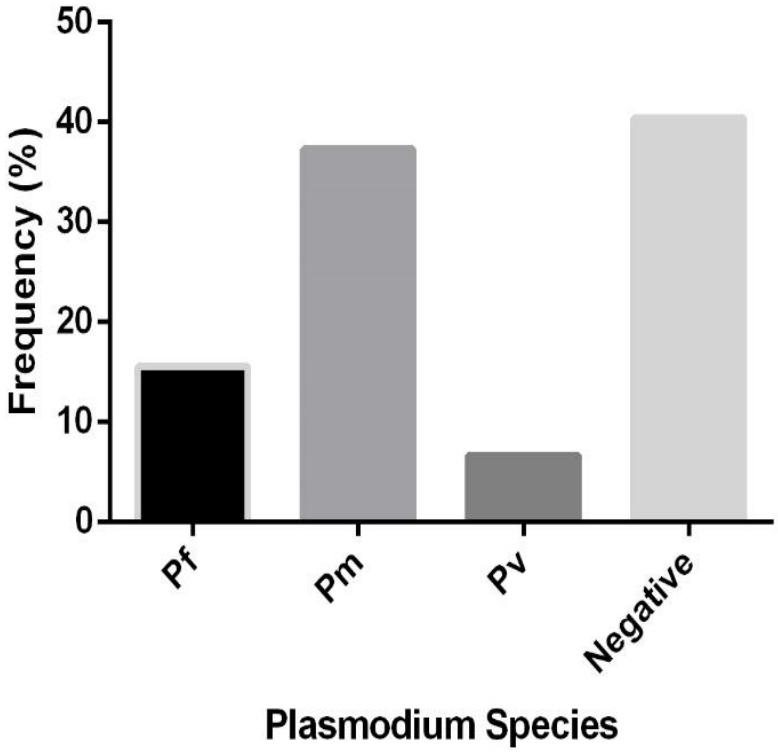



**Figure 45: *Plasmodium* species distribution in the Adult Population Cohort**

| Gene | Codon | Amino Acids Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| PfMDR1 (n=26) | 86 | N | Y | 26(100) | 0(0) | 0(0) |
| | 184 | Y | F | 20(76.92) | 6(23.08) | 0(0) |
| | 1069 | T | T | 25(96.15) | 1(3.85) | 0(0) |
| | 1114 | G | G | 25(96.15) | 0(0) | 1(3.85) |
| | 1143 | I | V | 25(96.15) | 0(0) | 1(3.85) |
| | 1157 | T(ACA) | T(ACG) | 25(96.15) | 1(3.85) | 0(0) |
| | 1243 | N | K | 25(96.15) | 1(3.85) | 0(0) |

**Table 18: Frequency (%) and number (no) of the *Pfmdr1* mutations in the Adult Population Cohort**

### 4.3.13 :Frequency (%) and number (no) of the *Pfcrt* mutations with Possible Haplotypes in the Adult Population Cohort

Total of 21*P.falciparum* samples were sequenced for mutations in the *Pfcrt* gene analysis. Table 19 showed the frequency of the various mutations at different codons assesed. All the samples sequenced had 100% wild type at codon 72. When assesed for mutations at codon 74, 76, and 75, it was observed that they all had 90.48 % wild –type and 2 (9.52 %). The resultant amino acid changes were all non synonymous. Three common haplotypes associated with chloroquine resistance are CVMNK, SVMNT and CVIET. When analysed for the presence of the said hyplotypes, 19 (90.48 %) had CVIET haplotype, 2 (9.52 %) had CVMNT while 0 (0) had 0 (0%). Non of the samples had heterozygote allele.

### 4.3.14: Frequency (%) and number (no) of the *Pfatpase6* mutations in the Adult Population Cohort

Table 20 showed the frequency and number of mutations in the pfatpase6 gene in the adult population cohort. Total of 7 samples were sucessfully sequenced and analysed for single nucletide polymorphisms (SNP). Mutations at codon 1723 had a frequency of 1 (14.28 %) mutants and 6 (85.71 %) wild type. Several known codons (243, 263, 431, 623 and 769) were also analysed for SNP, though they were all wild type.

### 4.3.15: Frequency (%) and number (no) of the *Pfkelch 13 gene* mutations in the Adult Population Cohort

13 samples were fully sequenced for assessment of single nucleotide polymorphisms (SNP) in *Pfkelch* 13 gene. Table 21 revealed codons that were assesed for SNP. Only 458 had mutations which was synonymous . The rest in the table had 100% wild type sequences.

Also, at codon 458, heterozygote allele was observed with frequency of 7.69 % of the entire sequences analysed for the same.

**Table 19: Frequency (%) and number (no) of the *Pfcrt* mutations with Possible**

| Gene | Codon | Amino Acids Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| Pfcrt n=21 | 72 | C | S | 21(100) | 0(0) | 0(0) |
| | 74 | M | I | 2(9.52) | 19(90.48) | 0(0) |
| | 75 | N | E | 2(9.52) | 19(90.48) | 0(0) |
| | 76 | K | T | 2(9.52) | 19(90.48) | 0(0) |
| Haplotypes | | | | | | |
| CVMNT n(%) | | | SVMNT n(%) | | CVIET n(%) | |
| 2(9.52) | | | 0(0) | | 19(90.48) | |

**Haplotypes in the Adult Population Cohort**

**Table 20: Frequency (%) and number (no) of the *Pfatpase6* mutations in the Adult Population Cohort**

| Gene | Codon | Amino Acids | | | | |
|---|---|---|---|---|---|---|
| | | Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
| Pfatpase6 n=7 | 1723 | I | V | 6(85.71) | 1(14.28) | 0(0) |
| | 243 | H | Y | 7(100) | 0(0) | 0(0) |
| | 263 | L | E | 7(100) | 0(0) | 0(0) |
| | 431 | E | K | 7(100) | 0(0) | 0(0) |
| | 623 | A | S | 7(1O0) | 0(0) | 0(0) |
| | 769 | S | N | 7(100) | 0(0) | 0(0) |

**Table 21: Frequency (%) and number (no) of the *Pfkelch 13 gene* mutations in the Adult Population Cohort**

| Gene | Codon | Amino Acids Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| Pfk13 n=13 | 458 | N(AAT) | N(AAC) | 12(92.31) | 0(0) | 1(7.69) |
| | 476 | M | I | 13(0) | 0(0) | 0(0) |
| | 493 | Y | H | 13(0) | 0(0) | 0(0) |
| | 539 | R | T | 13(0) | 0(0) | 0(0) |
| | 543 | I | T | 13(0) | 0(0) | 0(0) |
| | 580 | C | Y | 13(0) | 0(0) | 0(0) |

## 4.4 Overall Population

### 4.4.1 Overall Gender Distribution in Nnewi Population, South East Nigeria

The general gender distribution observed in the combined cohorts sampled in Nnewi, south East Nigeria were 38.19 % and 61.81 % for male and female respectively. This is also shown in figure 46 above. The combined cohorts includes Children, Pregnant women and mixed populations.

### 4.4.2 Overall Use of Mosquito Net in Nnewi, South East Nigeria.

The outlook of the use of mosquito net in Nnewi, South East Nigeria is as presented in Figure 47 below. 26.034% of the population said 'Always' in response to the use of mosquito nets. 65.693 % said 'Never' in response to the use of Mosquito nets, while 8.151 % said that they do use the net 'Sometimes'.

### 4.4.3 Overall Use of Mosquito Repellents in Nnewi, South East Nigeria

Figure 48 presents the picture of the attitude of the residents in Nnewi, South East Nigeria to the use of mosquito repellents. Only 15.45 % of the sampled population indicated to be using mosquito repellent 'Always'in their home while 59.49 % and 25.06 % repectively said 'Never' and 'Sometimes' in their response to the use of mosquito repellent.

### 4.4.4 Different Levels of Education Amongst the Population Sampled in Nnewi, South East, Nigeria

Figure 49 detailed the different levels of education of all the participants in Nnewi, South East, Nigeria. Structured questionaire was used to evaluate this while analysis was done with Graphpad Prism 6. The outcome showed that 121 had primary education. 352 had secondary education while 349 had  tertairy education.

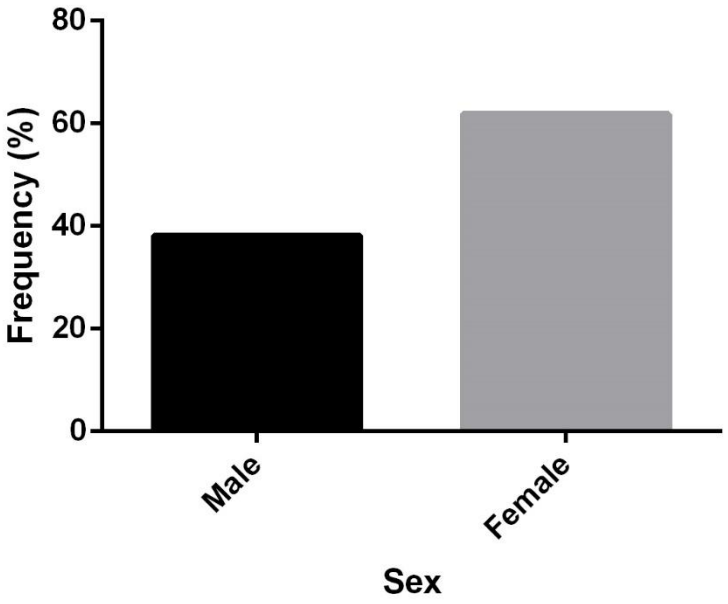



**Figure 46: Overall  Gender Distribution in  Nnewi Population, South East Nigeria**

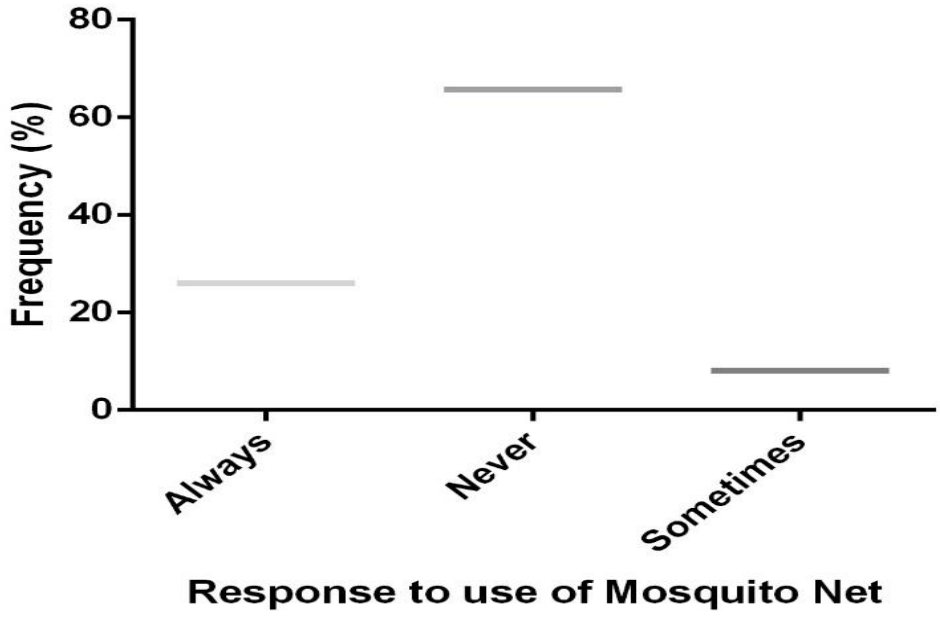



**Figure 47: Over All Use of Mosquito Net in Nnewi, South East Nigeria.**

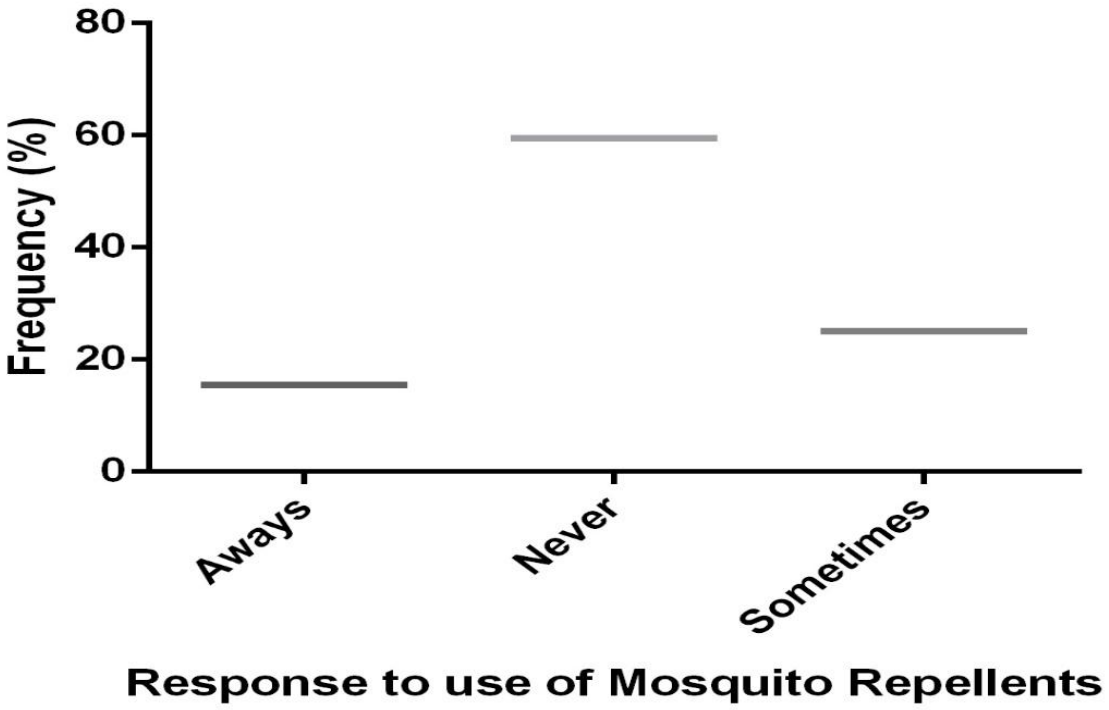



**Figure 48: Overall Use of Mosquito Repellents in Nnewi, South East Nigeria**

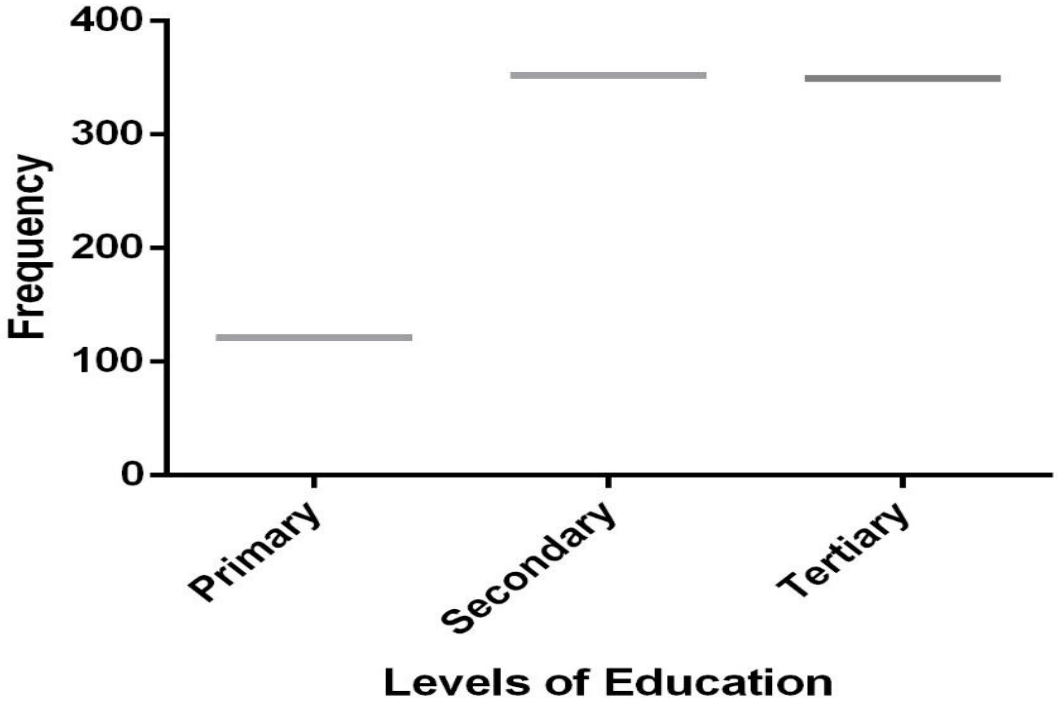



**Figure 49: Different Levels of Education Amongst the Population Sampled in Nnewi, South East, Nigeria**

### 4.4.5 OverAll Use of Mosquito Net with Respect to Levels of Education

Figure 50 detailed the frequency of mosquito net use with respect to highest level of education in all the cohorts sampled in Nnewi, South East Nigeria. A two way Anova was used to assess the statistical significance of the results. *P* value was set at $p \leq 0.05$. Within the response group with primary Education as the highest level of education, 37 persons said 'Always', 82 persons said 'Never' and 8 persons said that they 'Sometimes' use the mosquito net. In the response group that had secondary education as their highest level of education, 70 said 'Always' to the use of mosquito net, 251 said 'Never' to the use of mosquito net while 30 uses the net 'Sometimes'. In the resonse group that had Tertiary education as their highest level of education, 110 persons said 'Always', 208 persons said 'Never' while 30 persons indicated to be using the nets 'Sometimes'. The two way Anova results showed that only the row factors were statistcally significant ( $p = 0.0256$) while the column factor was not significat ( $p = 0.1622$).

### 4.4.6 Mean Parasite Density Across the Three Population Cohorts sampled in Nnewi, South East Nigeria.

Mean parasite densities of the three population cohorts sampled were compared using Anova, Brown-Forsythe test and Barlett's test. Graph pad prism 6 was used to run the analysis. *P* value was set at $p \leq 0.05$. The mean values of the various groups were 1205.51 ± 41.92, 2107.60 ± 66.79 and 1897.68 ± 65.69 for Adult group, Children group and Pregnant women group respectively (Figure 51). The children group had the highest mean parasite density followed by pregnant women group and finally the adult group. The analysis using the said tests gave varying p values of 0.2829, 0.8307 and 0.6330 for Anova, Brown-Forsythe test and Barlett's tests respectively. Following the set *p* value, they were statistically not significant.

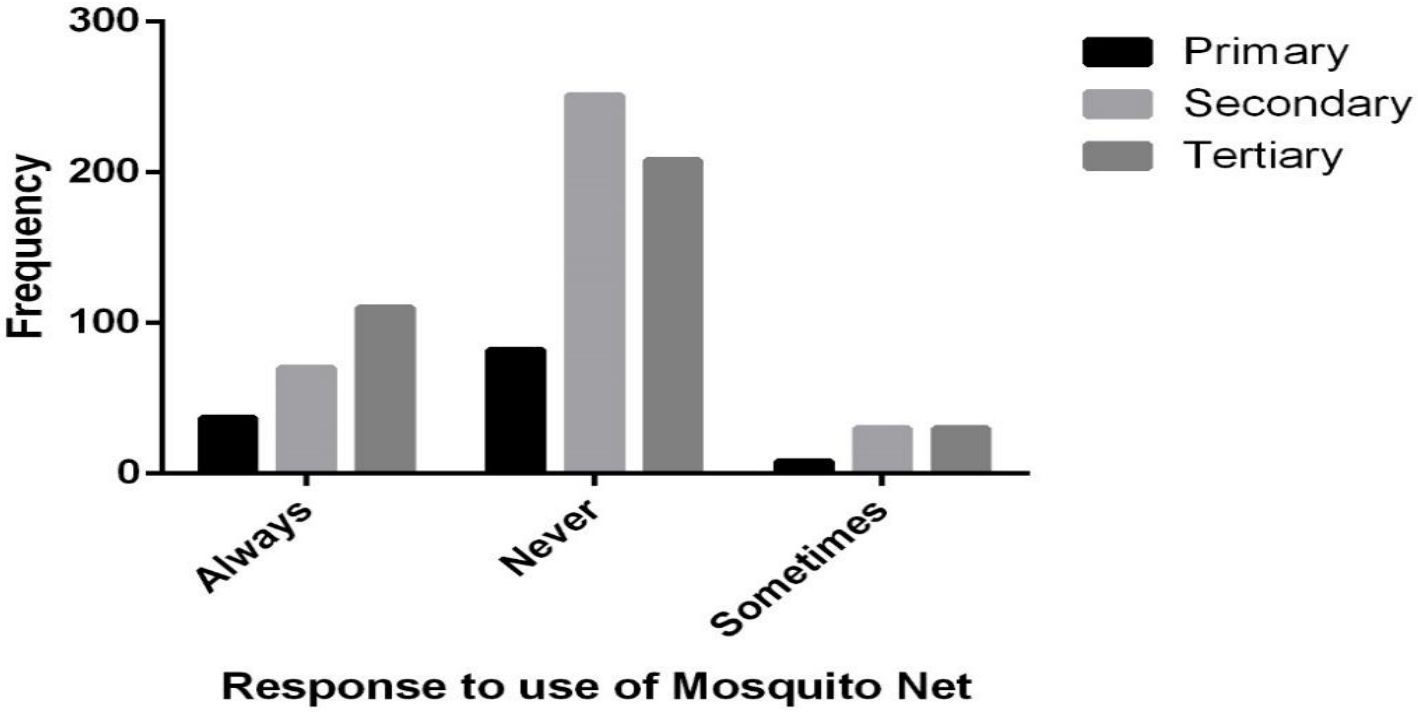
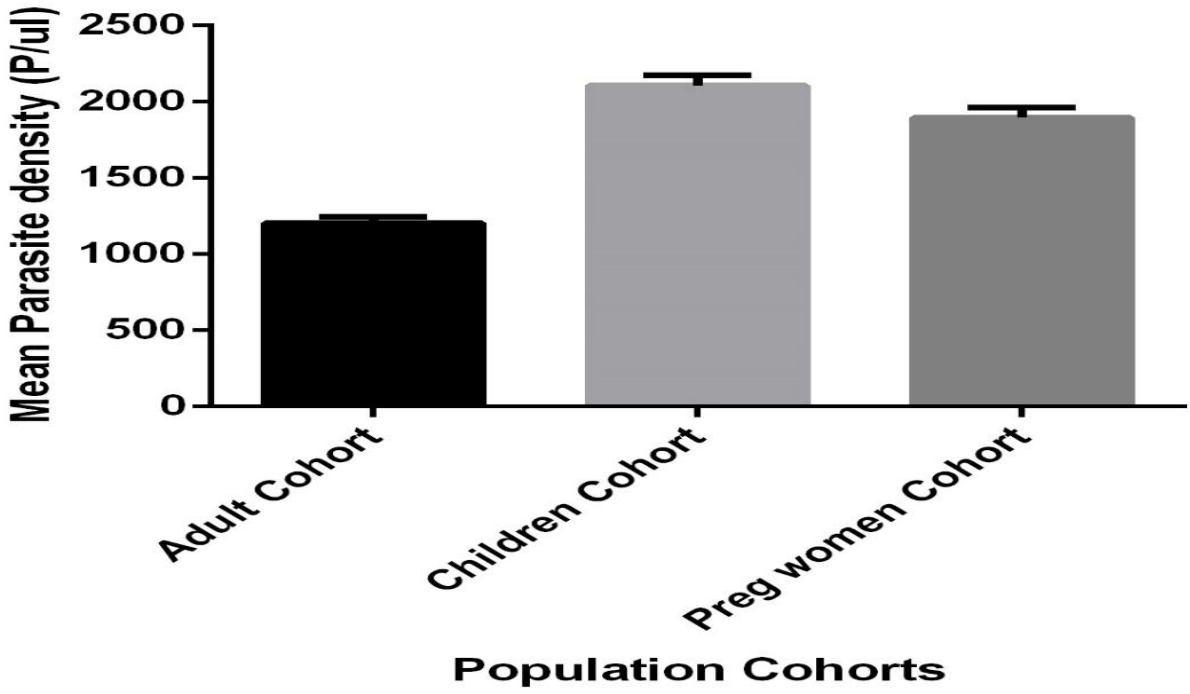



**Figure 50: OverAll Use of Mosquito Net with Respect to Levels of Education**

**Figure 51: Mean Parasite Density Across the Three Population Cohorts sampled in**




**Nnewi, South East Nigeria.**

### 4.4.7 Tukey's Mutiple Comparisons of the Mean Parasite Densities of the Three Population Cohorts sampled

Table 22 presents the results of the multiple comparisons of the mean parasite densities of the three population cohorts sampled. Confidence level was set at 95%. Mean differences of the various groups compared were as shown in Table 22. Importantly, they were all statistically significant ( 95% Cl).

### 4.4.8Overall Mean Parasite density with respect to the use of Mosquito Net

Mean parasite density was correlated with the use of mosquito net as shown in Figure 52 above. Anova, Brown – Forsythe and Bartlett's tests were used to compare the means of the parasite density of the various response groups in the population. P value was set at $p \leq 0.05$. The mean values of the various response groups were 1773.58 ± 72.43 (Always), 1502.05 ± 56.37 (Never) and 1788.66 ± 128.63 ( Sometimes). The analysis using the three test approches were statistically not significant with varying *p* values of 0.2829, 0.8307 and 0.6330 for Anova, Brown – Forsythe and Bartlett's tests respectively.

### 4.4.9 Overall Parasite Densities With Respect to Use of Mosquito Repellent

Mean parasite density was compared with the various responds groups in the use of mosquito repellant (Figure 53). Anova, Brown – Forsythe and Bartlett's tests were used for the analysis. P value was set at $p \leq 0.05$. It was observed that response group that said 'Always' had 1919.69 ± 107.69 Mean parasite density (P/µL). The response group that said 'Never'had 1666.92 ± 59.59 mean parasite density (P/µL), while the response group that said 'Sometimes' had 1573.59 ± 65.35 mean parasite density (P/µL). The analysis showed that the three tests used to compare the means of the diferent response groups had *p* values less than 0.05 ( $p = 0.0112$ for Anova).

**Table 22: Tukey's Mutiple Comparisons of the Mean Parasite Densities of the Three**

| Tukey's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? |
|---|---|---|---|
| Adult Cohort vs. Children Cohort | -902.1 | -1091 to -713.0 | Yes |
| Adult Cohort vs. Preg women Cohort | -692.2 | -879.5 to -504.9 | Yes |
| Children Cohort vs. Preg women Cohort | 209.9 | 12.37 to 407.5 | Yes |

**Population Cohorts sampled**

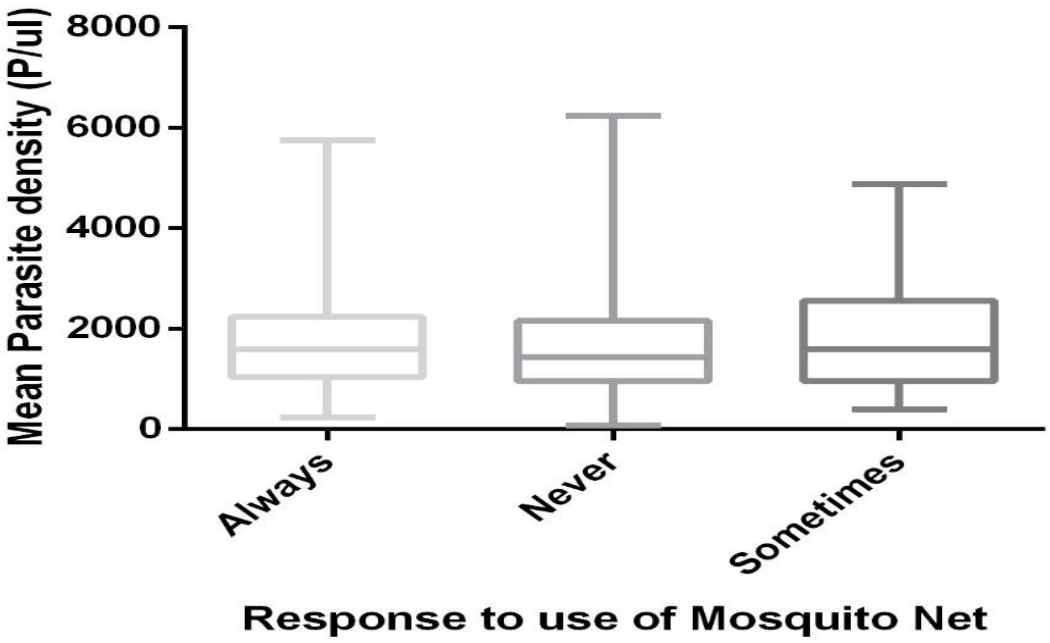



**Figure 52: Overall Mean Parasite density with respect to the use of Mosquito Net**

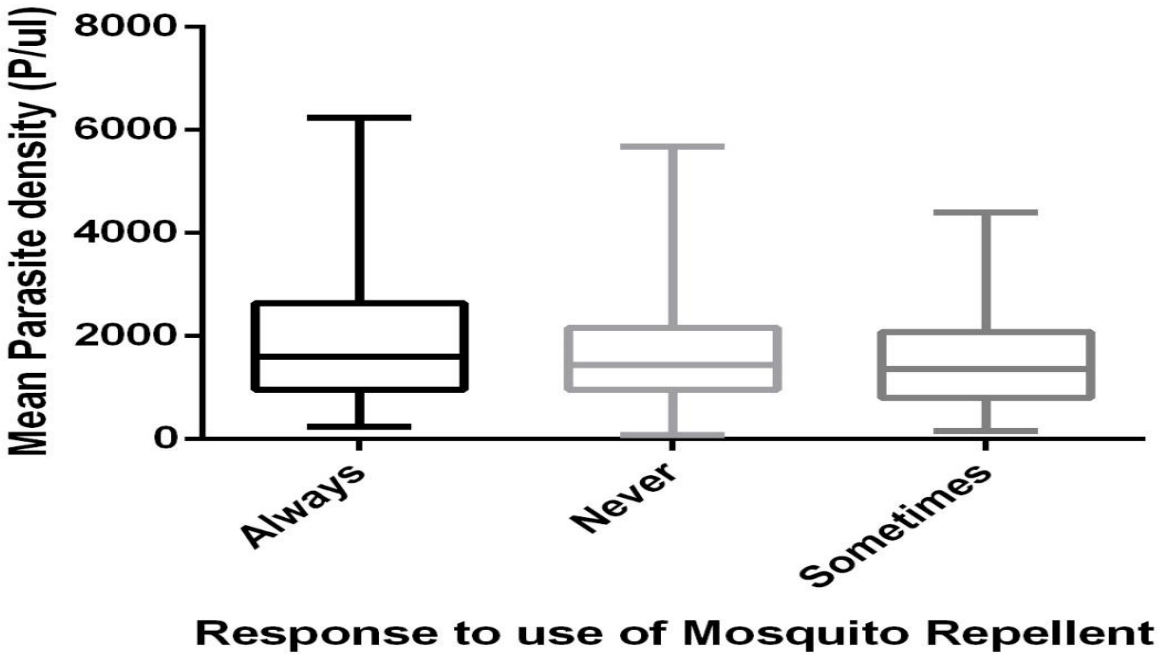



**Figure 53: Overall Parasite Densities With Respect to Use of Mosquito Repellent**

### 4.4.10Percentage Prevalence of *Plasmodium* Species Amongst Asymtomatic individuals in Nnewi District, South East Nigeria

The Percentage prevalence of *Plasmodium* species in Nnewi amongst asymtomatic individuals were analysed. The results is as presented in Figure 54 above. Polymerase chain reaction was used to reconfirm diagnosis made with microscopy during sample collection. Specific primers for each species were used in the genotyping. Figure 54 showed that out of 725 samples that were subjected to molecular genotyping, 103 (14.21%) were *Plasmodium falciparium* species, 170 (23.4 %) were *Plasmodium malariae*, 31( 4.27%) were *Plasmodium vivax* and 421( 58.07 %) were negative of the species analysed. *Plasmodium ovale* were not present in the samples analysed.

### 4.4.11 Distribution of Individuals with Single and Mixed infections of *Plasmodium* species in Nnewi District, SouthEast Nigeria.

Figure 55 above reflects the frequency of single and mixed infection observed in the three population cohorts sampled. Amongst the samples with single infection, *Plasmodium falciparum* were 64 (25.1 %) , *Plasmodium malariae* were 126 (49.41 %), *Plasmodium vivax* were 17(6.67 %). No record of *Plasmodium ovale*in the populations sampled. The figure also revealed some samples with mixed infection with more than one species of *Plasmodium.* Frequency of mixed infection present were 34 (13.33 %) for *Plasmodium falciparium + Plasmodium malariae,* 4 (1.57 %) for *Plasmodium falciparum + Plasmodium vivax,* 9 (3.53 %) for *Plasmodium malariae+ Plasmodium vivax,* and 1 (0.39 %) for *P. falciparum + P. malariae + P. vivax*

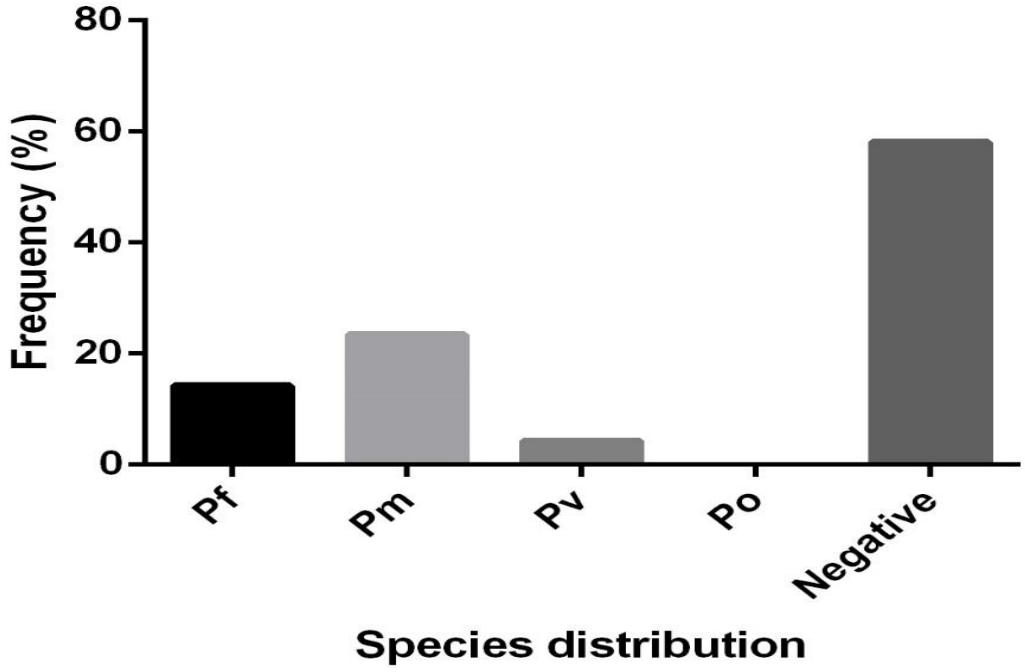



**Figure 54: Percentage Prevalence of *Plasmodium* Species Amongst Asymtomatic individuals in Nnewi District, South East Nigeria**

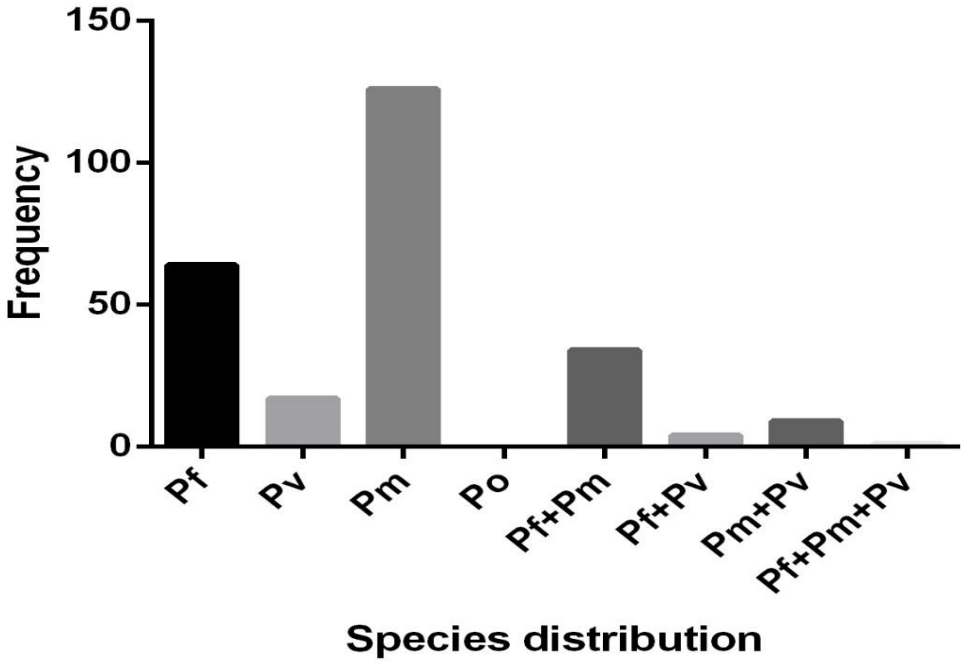



**Figure 55: Distribution of Individuals with Single and Mixed infections of *Plasmodium* species in Nnewi District, SouthEast Nigeria.**

### 4.4.12 Genetic diversity of *msp1, msp2, and glurp* measured as expected heterozygosity (HE)

Table 23above detailed the genetic diversity measured as expected heterozygosity. Expected Heterozygosity was calculated using the realtionship 'HE= [n/(n-1)] [(1-∑Pi2)]'. In children Cohorts, Msp-1 and Msp-2 had almost the same expected heterozygosity ( 0.57 and 0.59 respectively) while glurp had 0.33. Pregnant women had 0.13, 0.49 and 0.89 expected heterozygosity for Msp-1, Msp-2 and glurp respectively. Mixed Population had expected heterozygosity values of 0.59, 0.58 and 0.92 for Msp-1, Msp-2 and glurp respectively. Msp-1 and Msp-2 had almost the same values.

**Table 23:Genetic diversity of *msp1, msp2, and glurp* measured as expected heterozygosity (HE)**

| HE= [n/(n-1)] [(1-∑Pi2)] | | | |
|---|---|---|---|
| Gene | Children samples | Pregnant women | Adult Population |
| Msp 2 | 0.57 | 0.13 | 0.59 |
| Msp1 | 0.59 | 0.49 | 0.58 |
| glurp | 0.33 | 0.89 | 0.92 |

### 4.4.13: Type of Infection for Msp-1 and Msp-2 genes of *P.falciparum* Isolates from Nnewi, South East Nigeria

Table 24 detailed the type of infection for Msp-1 and Msp-2 genes for *P. falciparum* isolates from Nnewi, South East Nigeria. 88 % of samples positive for Msp-1 gene were monoclonal infection. MAD 20 was the predominant family present (78.57 %). Polyclonal infection was found in 12 % of the samples analysed for Msp-1 gene. Among the polyclonal infection, only RO33/MAD20 constituted the 12% observed. Monoclonal and Polyclonal infections for Msp-2 gene were 13 (44.83 %) and 16 (55.17 %) respectively. In monoclonal infections, 3D7 had the highest predominance [ 7(30.43 %)] while FC27 families were 5 ( 23.81 %). Among the polyclonal infections in MSP-2 gene, FC27/ 3D7 constituted the 55.17 % recorded.

In pregnant women population cohort, monoclonal and polyclonal infections were 8 (100 %) and 0 (0 %) respectively for Msp-1 gene. All the amplified gene products were all monoclonal, predominantly K1 (5) family and MAD20 (3) family. There were no visible amplification for RO33 family in the Msp-1 gene of *P. falciparum* from the pregnant women cohort. For the Msp -2 gene, monoclonal and polyclonal infections were 5 ( 38.46 % ) and 8 (61.54 % ) respectively. FC27 predominates in the monoclonal infections (33.33 %).

Monoclonal and polyclonal infections observed in the adult population cohort were 21 (70%) and 9 (30 %) respectively for Msp-1 gene of the *P. falciparum* present in the said population. All the three families of the msp-1 were identified. K1 had the highest frequency of 20 (71.43 %) of the samples analysed while MAD20 had only 1 (16.67 %). There were no monoclonal infections observed with RO33 family. Among the polyclonal infections, K1/MAD20 were 4 (11.76 %) while RO33/K1 and MAD20/RO33 were 4 (12.12%) and 1 (9.09 %). Trimorphic infections were not observed from the analysis.

**Table 24:Type of Infection for Msp-1 and Msp-2 genes of *P. falciparum* Isolates from Nnewi, South East Nigeria.**

| Chldren Cohort | | |
|---|---|---|
| Gene | Monoclonal infection n(%) | Polyclonal infection n(%) |
| MSP-1 | 22 (88) | 3 (12) |
| MSP-2 | 13 (44.83) | 16 (55.17) |
| Pregnant Women | | |
| MSP-1 | 8 (100) | 0 (0) |
| MSP-2 | 5 (38.46) | 8 (61.54) |
| Mixed Cohort | | |
| MSP1 | 21(70) | 9(30) |
| MSP2 | 4(20) | 16(80) |

### 4.4.14 Overall Proportions of various families of Msp 1, Msp 2 and Glurp gene of *P. falciparum* Isolates from Nnewi, South East Nigeria.

Table 25 detailed the genetic diversities of the *Plasmodium falciparum* present in the population sampled. All samples positive for *P. falciparum* were genotyped for genetic diversity using MSP 1, MSP 2 and Glurp markers by Nested PCR.

In the Children cohort population, 28(58.31 %) were positive for Msp-1 while 44(91.63 %) were positive for Msp-2. 30 (62.5 %) were positive for Glurp. The multiplicity of infection observed were 1.36, 2.3 and 1.06 for Msp-1, Msp-2 and Glurp respectively. In Msp-1, K1, RO33, and MAD20 were all identified with overall frequency 28. MAD 20 family was predominant as it was identified in 29.16 % (14/48) of the *P. falciparum* from children samples. In Msp-2, both 3D7 and FC27 allelic families were identified with overall frequency of 44. 3D7 was predominant as it was identified in 47.9 % (23/48) of the *P.falciparum* from children samples.

In the pregnant women cohort, 8 ( 20 % ) were positive for Msp-1 while 21 (52.5 %) were positive for Msp-2. Glurp had 19 (95 %) that were positive. The multiplicity of infection ( MOI) observed were 1, 2.51 and 1.84 for Msp-1, Msp-2 and Glurp respectively. In Msp-1, only K1 and MAD 20 were identified. Here, K1 had the highest predominance with 25 % (5/20) of the samples genotyped. In Msp-2, both 3D7 and FC27 were identified with 3D7 having the highest frequency (60%).

Also, in the adult population cohort, 39 (38.2 %) were positive for Msp-1 while 37 (57.8 %) were positive for Msp-2. Glurp had 26 (76.4 %) positive samples. The multiplicity of infections observed were 1.1, 2.4 and 1.3 for Msp-1, Msp-2 and glurp respectively. In Msp-1, K1 had the highest predominance (82.2 %) while in Msp-2, 3D7 had the highest

predominance (58.8 %). Frequency of 26 (76.4 %) were observed of the samples genotyped for Glurp.

**Table 25: Overall Proportions of various families of Msp 1, Msp 2 and Glurp gene of *P. falciparum* Isolates from Nnewi, South East Nigeria.**

| Marker | Children Cohort n(%) | Pregnant women Cohort n (%) | Adult Population Cohort n(%) |
|---|---|---|---|
| Msp 1 families | | | |
| K1 | | | |
| n (%) | 5(10.4) | 5 (25) | 28(82.3) |
| Genotypes | 1 | 1 | 2 |
| MAD 20 | | | |
| n (%) | 14 (29.16) | 3 (15) | 6(17.6) |
| Genotypes | 2 | 1 | 1 |
| RO33 | | | |
| n (%) | 9 (18.75) | 0(0) | 5 (14.2) |
| Genotypes | 1 | 0 | 1 |
| MOI for Msp 1 | 1.36 | 1 | 1.1 |
| MSP 2 families | | | |
| FC27 | | | |
| n (%) | 21 (43.73) | 9(45) | 17 (50) |
| Genotypes | 6 | 5 | 3 |
| 3D7 | | | |
| n (%) | 23 (47.9) | 12 (60) | 20(58.8) |
| Genotypes | 4 | 4 | 5 |
| MOI for Msp 2 | 2.3 | 2.51 | 2.4 |
| Glurp | | | |
| n (%) | 30 (62.5) | 19 (95) | 26 (76.4) |
| Genotypes | 2 | 3 | 3 |
| MOI for Glurp | 1.06 | 1.84 | 1.3 |

### 4.4.15 Alignment of the deduced Amino Acid Sequences Encoding for MSP2 (3D7) Revealing Six Sequence variants.

Some bands of 3D7 with equal base pairs as revealed in the gel were cut, purified using GE Health purification kit and ultimately sequenced. Geneious software were used to convert the said sequences to amino acids. Figure 56 above is the alignment of the amino acid sequences. The alignment was done with the reference sequences of MSP2 in 3D7 family of *Plasmodium falciparum*. The alignment revealed great variations between the amino acids compositions of the six sequences compared.

### 4.4.16 Frequency (%) and number (no) of the *Pfmdr1* mutations in the entire Populations sampled at Nnewi, South East Nigeria

The *Pfmdr1* gene was sucessfully sequenced in 82 samples amongst the *P. falciparum* found in the three populations sampled at Nnewi, South East Nigeria. The results for *pfmdr1* polymorphisms were shown in Table 26. The frequency of the N86Y was 75 (91.46 % ) for the wild type and 7 (8.54 %) for mutants. Y184F had frequency of 55 ( 67.07 % ) for the wild type and 19 (52.78 % ) for mutants. Samples possessing Heterozygous alleles were also observed in some samples. 3 (3.66 % ) of the samples had heterozygous alleles at codon 184. Also at codons 1066,1090, 1114, 1143 of the *pfmdr1* genes sequenced, 1 (1.22 % ) of the samples had heterozygote alleles respectively. Table 24 detailed other uncommon mutations observed in the sequences of the gene sequenced. Other new mutations observed were as shown in the Table 26. At codon 1157, 1(1.22 %) of the sequenced samples had synonymous mutation. All the other mutations observed were non synonymous.

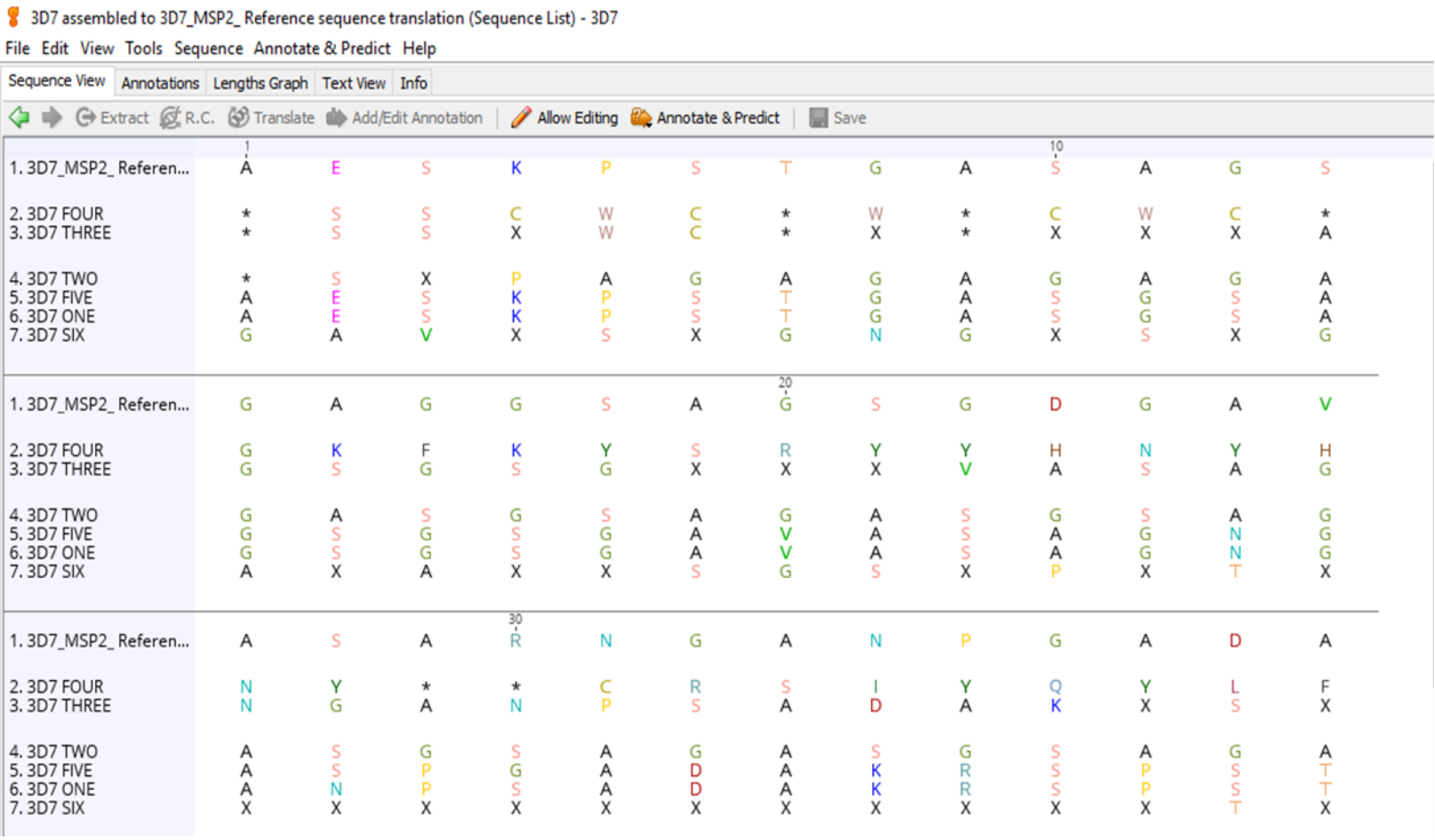



**Figure 56: Alignment of the deduced Amino Acid Sequences Encoding for MSP2 (3D7) Revealing Six Sequence variants.**

**Table 26: Frequency (%) and number (no) of the *Pfmdr1* mutations in the entire Populations sampled at Nnewi, South East Nigeria**

| Gene | Codon | Amino Acids | | | | |
|---|---|---|---|---|---|---|
| | | Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
| PfMDR1 (n=82) | 86 | N | Y | 75(91.46) | 7(8.54) | 0(0) |
| | 158 | Y | H | 81(98.78) | 1(1.22) | 0(0) |
| | 184 | Y | F | 55(67.07) | 24(29.27) | 3(3.66) |
| | 74 | F | L | 81(98.78) | 1(1.22) | 0(0) |
| | 164 | S | S | 81(98.78) | 1(1.22) | 0(0) |
| | 1104 | K | E | 81(98.78) | 1(1.22) | 0(0) |
| | 1004 | Y | H | 81(98.78) | 1(1.22) | 0(0) |
| | 1066 | S | P | 81(98.78) | 0(0) | 1(1.22) |
| | 1069 | T | T | 80(97.56) | 2(2.44) | 0(0) |
| | 1077 | A | A | 81(98.78) | 1(1.22) | 0(0) |
| | 1090 | A | A | 81(98.78) | 0(0) | 1(1.22) |
| | 1106 | N | S | 81(98.78) | 1(1.22) | 0(0) |
| | 1117 | I | V | 81(98.78) | 1(1.22) | 0(0) |
| | 1176 | R | G | 81(98.78) | 1(1.22) | 0(0) |
| | 1122 | I | T | 81(98.78) | 1(1.22) | 0(0) |
| | 1129 | K | R | 81(98.78) | 1(1.22) | 0(0) |
| | 1137 | S | S | 81(98.78) | 1(1.22) | 0(0) |
| | 1246 | D | Y | 79(96.34) | 3(3.66) | 0(0) |
| | 1114 | G | G | 81(98.78) | 0(0) | 1(1.22) |
| | 1143 | I | V | 81(98.78) | 0(0) | 1(1.22) |
| | 1157 | T(ACA) | T(ACG) | 81(98.78) | 1(1.22) | 0(0) |
| | 1154 | K | K | 81(98.78) | 1(1.22) | 0(0) |
| | 1224 | T | T | 81(98.78) | 1(1.22) | 0(0) |
| | 1243 | N | K | 81(98.78) | 1(1.22) | 0(0) |
| | 1244 | R | G | 81(98.78) | 1(1.22) | 0(0) |

**4.4.17 Frequency (%) and number (n) of the *pfcrt* gene Mutations with Posible Haplotypes in Nnewi, South East Nigeria.**

Total of 55 *P. falciparum* samples were sequenced for mutations in the *Pfcrt* gene analysis. Table 27 showed the frequency of the various mutations at different codons assesed. All the samples sequenced had 100% wild type at codon 72. When assesed for mutations at codon 74, 76, and 75, it was observed that they all had 3 (5.45 %) wild –type and 52 (94.54 %). The

resultant amino acid changes were all non synonymous. Three common haplotypes associated with chloroquine resistance are CVMNK, SVMNT and CVIET. When analysed for the presence of the said hyplotypes, 42 (76.36 %) had CVIET haplotype, 3 (5.45 %) had CVMNT while 0 (0) had SVMNT. Non of the samples had heterozygote allele.

### 4.4.18 Frequency (%) and number (no) of the *Pfkelch 13* mutations in the entire Populations sampled at Nnewi, South East Nigeria

36 samples were fully sequenced for assessment of single nucleotide polymorphisms (SNP) in *Pfkelch* 13 gene. Table 28 revealed codons that were assesed for SNP. Most of the mutations observed were synonymous with exception of mutation observed at codon 547 with non synonymous changes in the amino acids. Most of the known mutations reported some other places (476, 493, 539, 543 and 580) were absent with wild type sequences. At codon 458, heterozygote allele was observed with frequency of 2.78 % of the entire sequences analysed for the same.

| Gene | Codon | Amino Acids | | | | |
|---|---|---|---|---|---|---|
| | | Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
| Pfcrt (n=55) | 72 | C | S | 55(100) | 0(0) | 0(0) |

| | | | | | |
|---|---|---|---|---|---|
| 74 | M | 1 | 3(5.45) | 52(94.54) | 0(0) |
| 75 | N | E | 3(5.45) | 52(94.54) | 0(0) |
| 76 | K | T | 3(5.45) | 52(94.54) | 0(0) |

| Haplotypes | | |
|---|---|---|
| CVMNK n(%) | SVMNT n(%) | CVIET n(%) |
| 3(5.45) | 0(0) | 42(76.36) |

**Table 27: Frequency (%) and number (n) of the *pfcrt*gene Mutations with Posible Haplotypes in Nnewi, South East Nigeria.**

**Table 28: Frequency (%) and number (no) of the *Pfkelch 13* mutations in the entire Populations sampled at Nnewi, South East Nigeria.**

| Gene | Codon | Amino Acids | | | | |
|---|---|---|---|---|---|---|
| | | Wild-type/Mutant | | Wild-type n (%) | Mutants n(%) | Heterozygote n(%) |
| Pfk13 (n=36) | 458 | N(AAT) | N(AAC) | 35(97.22) | 0(0) | 1(2.78) |
| | 510 | V (GTG) | V (GTA) | 35(97.22) | 1(2.78) | 0(0) |
| | 515 | R (AGA) | R(AGG) | 35(97.22) | 1(2.78) | 0(0) |
| | 547 | D(GAT) | G(GGT) | 35(97.22) | 1(2.78) | 0(0) |
| | 613 | Q(CAA) | Q(CAG) | 35(97.22) | 1(2.78) | 0(0) |
| | 688 | E(GAA) | E(GAG) | 35(97.22) | 1(2.78) | 0(0) |
| | 476 | M | I | 36(100) | 0(0) | 0(0) |
| | 493 | Y | H | 36(100) | 0(0) | 0(0) |
| | 539 | R | T | 36(100) | 0(0) | 0(0) |

| | | | | | |
|---|---|---|---|---|---|
| 543 | I | T | 36(100) | 0(0) | 0(0) |
| 580 | C | Y | 36(100) | 0(0) | 0(0) |

### 4.4.19 Frequency (%) and number (no) of the *Pfatpase6* mutations observed in Nnewi, South East Nigeria

Table 29 showed the frequency and number of mutations in the *pfatpase6* gene in all the populations . Total of 24 samples were sucessfully sequenced and analysed for SNP. Mutations at codon 1723 had a frequency of 1 (4.17 %) mutants and 23 (95.83 %) wild type. At codons 569 and 639, frequency of wild type and mutants were 91.67 %, 8.33 % and 91.67 %, 4.17 % respectively. Heterozygosity was observed at codon 639 with frequency of 4.17 %. Several known codons (243, 263, 431, 623 and 769) were also analysed for SNP, but they were all wild type.

### 4.4.20 Phylogenetic Anaylysis of Pfcsp isolates recovered from Nnewi, Southeast Nigeria

Figure 57 detailed the phylogenetic analysis of the CSP sequences. Two main clusters were

| Gene | Codon | Amino Acids |
|---|---|---|

observed. Various distance values were also indicated.

### 4.4.21 Sequence diversity/ variations in the central repeat region of PfCSP of some selected Isolates.

Figure 58 above is the alignment of the nucleotide sequences. The alignment was done with the reference sequences of CSP in 3D7 family of *Plasmodium falciparum*. The alignment revealed great variations between the nucleotide sequences of the isolates.

### 4.4.22 Sequence diversity/ variations in the T-cell (Th3) epitopes of CSP of some selected Isolates.

Figure 59 above is the alignment of the nucleotide sequences of the T-cell epitopes of the CSP. The alignment was done with the reference sequences of CSP in 3D7 family of *Plasmodium falciparum*. The alignment revealed minor variations between the nucleotide sequences of the isolates and that of the reference gene.

**Table 29: Frequency (%) and number (no) of the *Pfatpase6* mutations observed in Nnewi, South East Nigeria**

| | | Wild-type/Mutant | | Wild-type n (%) | Mutants n (%) | Heterozygote n(%) |
|---|---|---|---|---|---|---|
| Pfatpase6 n=24 | 569 | N(AAT) | K(AAA) | 22(91.67) | 2(8.33) | 0(0) |
| | 639 | G(GGC) | D(GAC) | 22(91.67) | 1(4.17) | 1(4.17) |
| | 243 | H | Y | 24(100) | 0(0) | 0(0) |
| | 263 | L | E | 24(100) | 0(0) | 0(0) |
| | 431 | E | K | 24(100) | 0(0) | 0(0) |
| | 623 | A | S | 24(100) | 0(0) | 0(0) |
| | 769 | S | N | 24(100) | 0(0) | 0(0) |
| | 17 | I | V | 23(95 | 1(4.1 | 0(0) |

23 .83) 7)

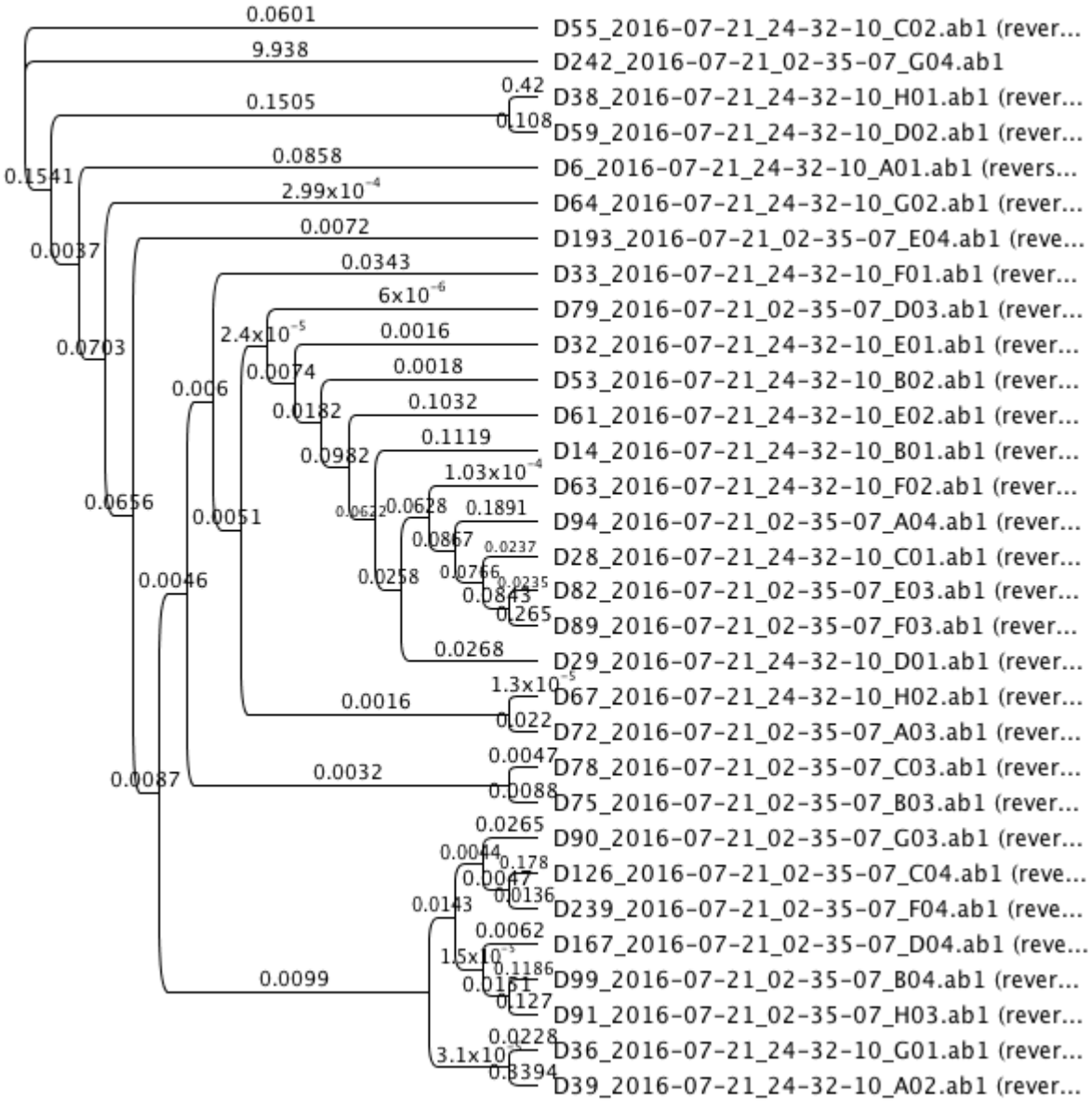



**Figure 57: Phylogenetic Anaylysis of Pfcsp isolates recovered from Nnewi, Southeast Nigeria**

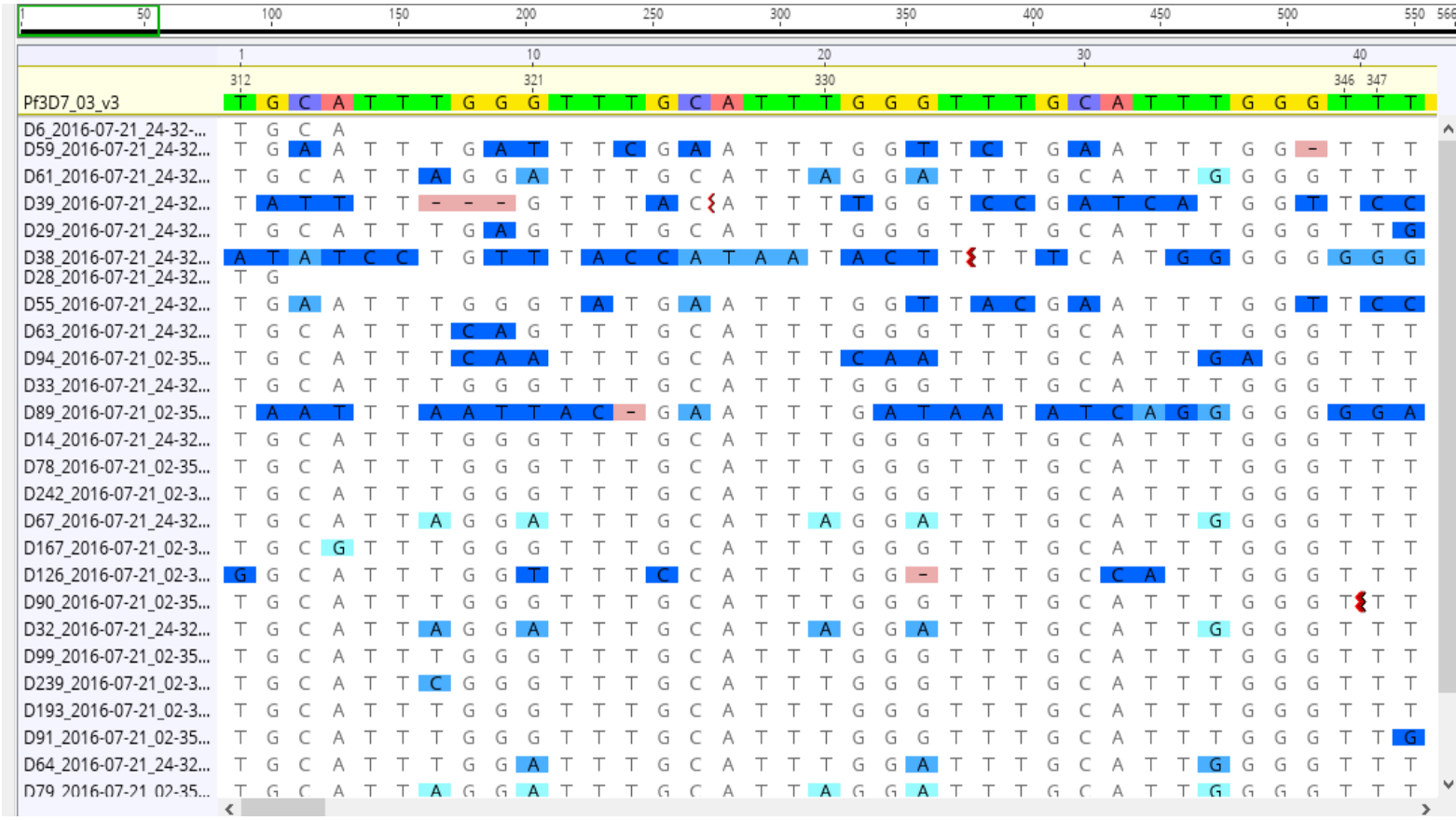



**Figure 58: Sequence diversity/ variations in the central repeat region of PfCSP of some selected Isolates.**

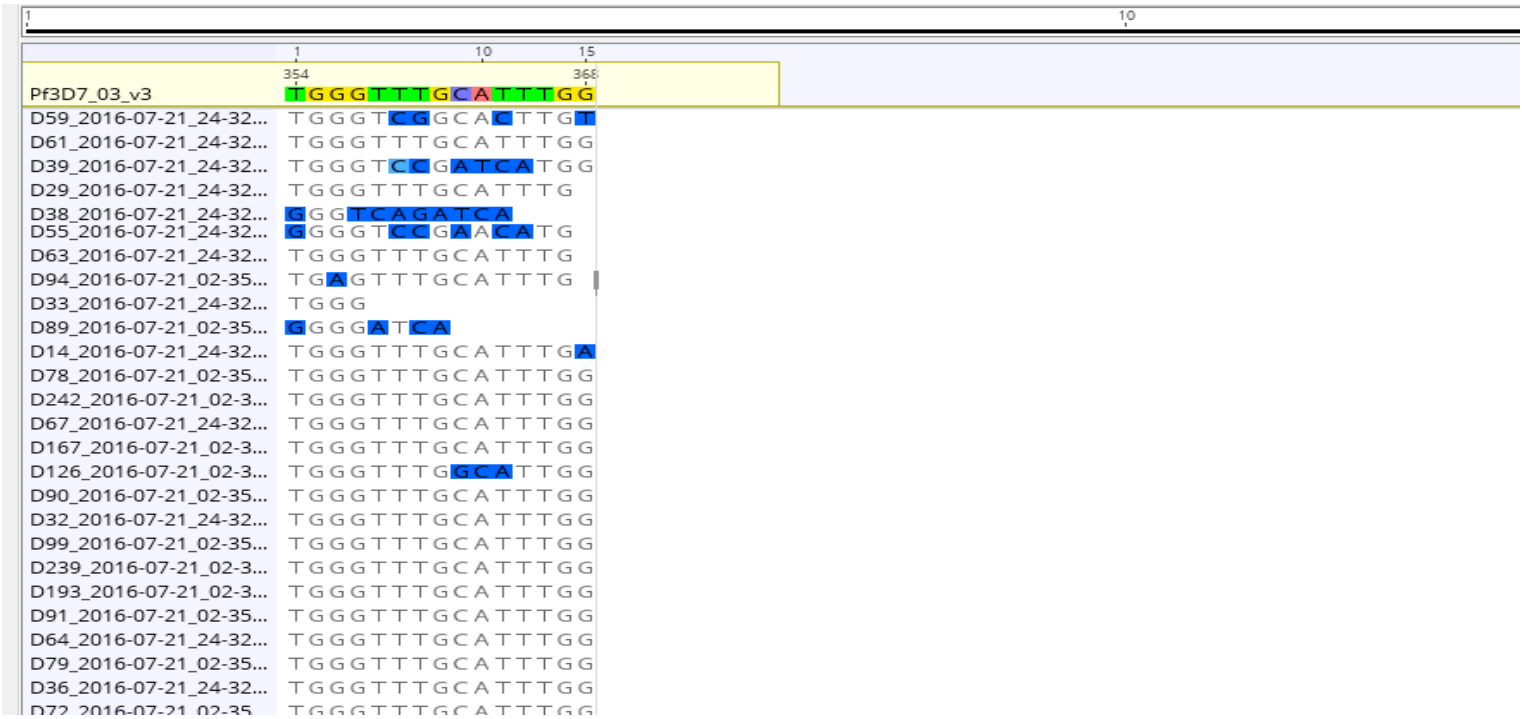



**Figure 59: : Sequence diversity/ variations in the T-cell (Th3) epitopes of CSP of some selected Isolates.**

### 4.4.23 Sequence diversity/ variations in the T-cell (Th2) epitopes of CSP of some selected Isolates.

Figure 60 is the alignment of the nucleotide sequences of the T-cell epitopes of the CSP. The alignment was done with the reference sequences of CSP in 3D7 family of *Plasmodium falciparum*. The alignment revealed minor variations between the nucleotide sequences of the isolates and that of the reference gene.

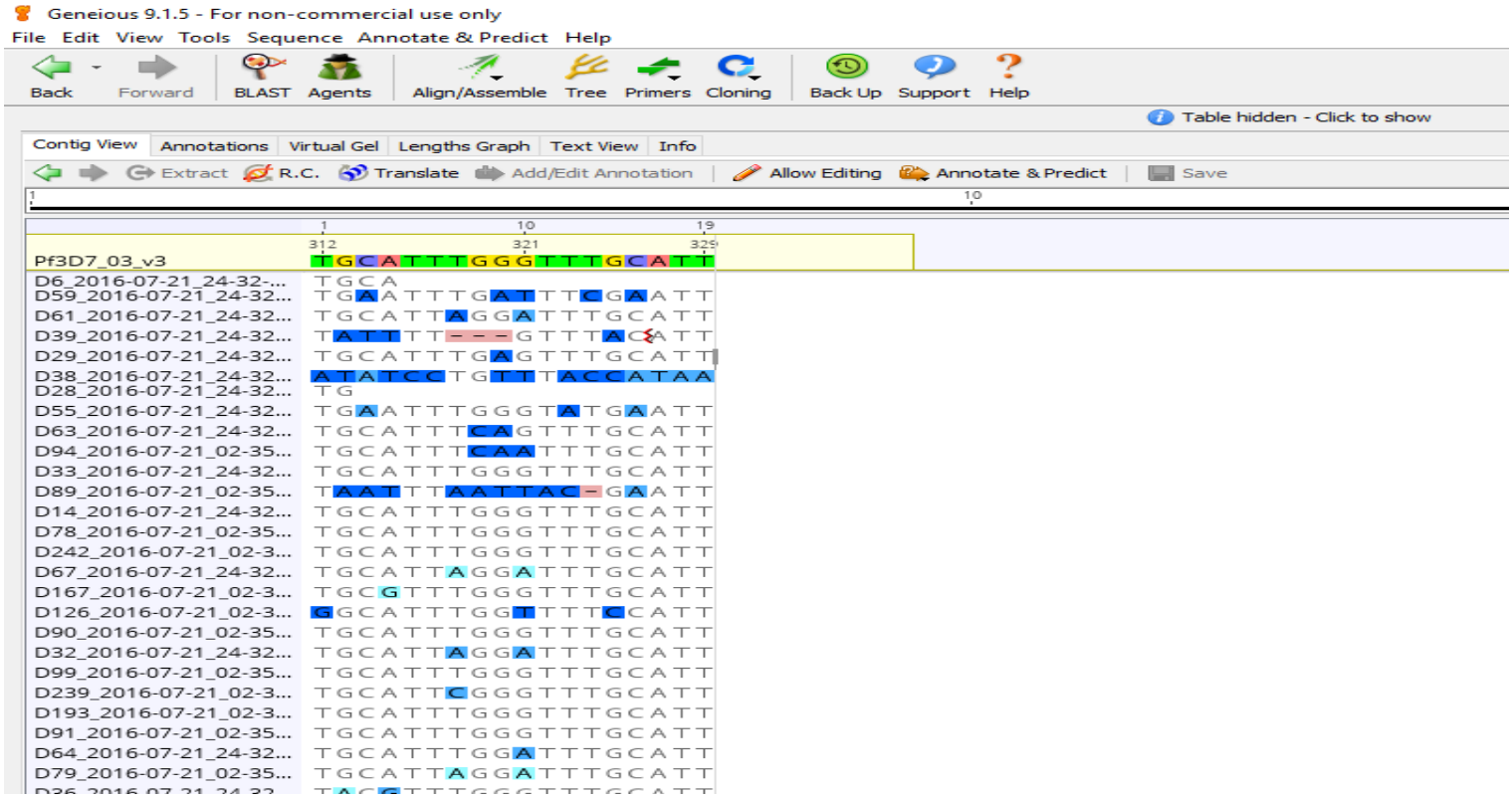



**Figure 60: Sequence diversity/ variations in the T-cell (Th2) epitopes of CSP of some selected Isolates.**

# CHAPTER FIVE

# DISCUSSION, CONCLUSION, RECOMMENDATION AND CONTRIBUTION TO KNOWLEDGE

## 5.1 Discussion

### 5.1.1 Response to Use of Mosquito Nets and Indoor Residual Repellents

In this study, the use of mosquito nets and repellents against mosquitos were evaluated. Amongst the three population cohorts evaluated, majority of the individual population responded 'Never' in their various responses to the use of mosquito nets and repellents.Children population (from mothers), pregnant women and adult population had 59.6 %, 59.46 % and 75.72 % respectively as the percentage of persons who indicated 'Never' in their use of mosquito net. The remaining percentages were shared between those who indicated 'Always' and those who use the nets 'Sometimes'. Pregnancy brings down immunity which predisposes many pregnant women to malaria infections and thereby increasing the risk of illness, anaemia severe disease and death (WHO, 2016). For the unborn child, maternal malaria increases the risk of abortion, stillbirth, premature delivery and low birth weight (leading cause of child mortality) (WHO, 2016). Insecticides-treated nets reduce mortality and morbidity due to malaria, but as revealed from this work, its use is limited. Similar observation was made in the in-house use of mosquito repellents in this study. Hindrances to usage of mosquito nets and repellents could be ignorance regarding malaria transmission and prevention (Sissoko *et al*., 2005). More than that, many who are in the knowing of the aforementioned believe that it's not convenient following the prevailing temperature in the region.Onwujekwu in his early works in 2000 at Enugu State Nigeria, discovered that there were good knowledge about malaria and the use of mosquito nets to prevent it (Onwujekwe *et al*., 2000). In his work, he found that many had knowledge of the

use of the mosquito nets,but few (8 %) had the nets in their possession. Also, in another work by Edelu in the same Enugu State, Nigeria, awareness and use of insecticide – treated bed nets among children attending outpatient clinic were evaluated. He discovered that 80 % of the mothers interviewed were aware of ITN, but only 26.1 % use it for their children (Edelu *et al*., 2010). In western Kenya, Sophia and colleagues sampled ownership, use and quality of mosquito nets. He found out that from 670 households surveyed, 95 % owned at least one net while only 59 % uses the net at night (Githinji *et al*., 2010). His findings partly deviated from most of the responses from Nigeria, as above 50 % uses the net beyond just being in possession of it. Also in Ethiopia, more than 50% of the population sampled uses the nets beyond possession (Baume *et al*.,2009). It could be that why Nigeria reports below 50 % is that use of the mosquito nets had not yet received good promotion, and/or the fund made available for the nets had been misappropriated by those in charge.In a review of selected literature from Pubmed, carried out by Singh*et al* on ownership and use of insecticides-treated nets during pregnancy in sub Saharan Africa in October 2012, they discovered that ownership of ITNs varied from 3 % to more than 80 %. In their work, educational level, knowledge of malaria, community involvement, socio-economic status and parity were highlighted as the main determinants (Sing *et al*., 2013). Very similar to what is obtainable from different persons in Nigeria who responded differently on the use of mosquito nets, the review underscored reasons for non-use of the mosquito nets despite being in possession of it as lack of supply of the nets, discomfort, problems of hanging up the nets and lack of space.

In the present work, use of mosquito nets was evaluated with respect to highest level of education of participants. The outcome revealed that majority of the participants with tertiary education do not use the mosquito nets. When analysed with anova, the value was found to be significant. It is very clear that the use of mosquito nets is not dependent on one's educational level. In Nnewi, south East Nigeria, the major reason for non-use of ITNs was discomfort as

a result of the hot temperate region. This is different from the reasons given in a similar work in Ethiopia (Baume *et al*., 2009), where reasons indicated include few mosquitos around or malaria being not a serious problems. Some also said that the ITN is no longer effective. The present findings were also in support of similar work by Ankomah *et al*. in 2012, where they discovered that levels of education had no impact on the use of mosquito nets in Nigeria

(Ankomah *et al.*, 2012).

The present study also evaluated their use of mosquito repellents. The results showed that only 15.45 % uses mosquito repellents in their house. This encompasses both indoor residual spraying and any other form of spraying that would stop the activities of mosquitos. This outcome may be as a result of many reasons which could be some being allergic to some kind of chemicals, cost of unending spending on the spray without definitive solution and irritation of some chemicals. Similar work to this, was carried out by Onwujekwe *et al.*in 2014 where there was a high levels use of both indoor spray and aerosol spray in Enugu State, Nigeria (Onwujekwe *et al*., 2014). This is in contrast to the present work. This may be due to differences in the activities in the two states assessed. Where as in Enugu, many persons living there are civilized, Nnewi is majorly business men that deals on motor cycle spare parts with probable less knowledge about malaria control.

### 5.1.2 Mean Parasite Density

Mean parasite density of each population cohorts were assessed and effects of age, use of mosquito nets and repellents were determined. On the face value (not statistically significant), children population had the highest mean parasite density when compared with pregnant women population and adult population (Figure 41). This could be due to non-immunity to the disease in some children. Also, those with partial immunity may not have strong antibodies that could combat with the parasite, hence proliferating within their body without

any opposition. Following some analysis to correlate the effect of age on the mean parasite density, the outcome was not statistically significant. This is very similar to the research carried out by Kolawole *et al*.in 2014, where they discovered that parasite densities of various age ranges from infant through adults were not statistically significant (Kolawole *et al*., 2014). Also in another work by Olusegun Akanbi in 2015, he compared the parasite densities of various age ranges of children under five years. He discovered that children in the group within the age range of 4-5 years had significantly higher parasite density compared with other age range of 0-1 and 2-5 (Akanbi*et al*., 2015). Similar trend was observed in the present study though not statistically significant. Research had revealed that genetics is a major determinant of parasite density, not age. In a work by Pullan *et al*.(2010) on 'Heritability of *Plasmodium*Parasite Density in a Rural Ugandan Community' they were able toobserve that genetic effects are the main determinant of parasite density in children in the population studied (Pullan *et al.,* 2010). Infact, as earlier as 2003, Flori *et al.* had linked *Plasmodium falciparum* blood infection levels and chromosome 5q31-q33 (Flori *et al*., 2003). The works concludes that Chromosome 5q31-q33 contains genes controlling blood infection levels. There are other possibilities that parasite density could be linked to parasite genetics and hosts immunity.

The present work also looked at the effect of use of mosquito nets and repellents on the parasite density of the populations sampled. The results showed that the individuals that used either mosquito nets or mosquito repellents had higher values of mean parasite density compared to those who were not using them (Figure 34 and Figure 35).While that of those that uses mosquito repellents were statistically significant, those that used mosquito nets were not. It would be naturally expected that those that used those preventive tools should have less parasite density, but here, opposite is the case. This still points to so many other factors that would be responsible for this level of infection. As discussed above, host genetics and

the parasite density could be the determinant factor to this. Some works had been done in the past to assess the relationship between parasitaemia and use of mosquito nets. Such is the work by Iwuafor *et al.*, 2016 titled 'Malaria parasitaemia and the use of insecticide – treated nets (INTs) for malaria control amongst under-5 year old children in Calabar, Nigeria'. They found out that there is no statistically significant reduction in malaria parasitaemia with the use of mosquito nets among the under -5 children (Iwuafor *et al*., 2016). Conversely, some works had shown significant reduction in the parasitaemia level as a result of mosquito net use (Apinjoh *et al*., 2015; Lim *et al*., 2011). The results obtained in this present work could be as a result of outdoor transmissions and resistance of vectors to insecticide- treated nets. Also, in a work by Kuile*et al*., 2003, it was observed that there is a reduction of malaria in pregnant women who used Permethrin- treated bed nets. Their findings specify that only women in gravidae 1-4 had the beneficial impact. In gravidae five and six, no such impact was observed (Kuile *et al*., 2003).

### 5.1.3 Distribution of *Plasmodium* species in the Region

The present work assessed the prevalence of *Plasmodium* species in circulation in Nnewi, south eastern Nigeria using nested PCR. 14.21% (103) were found to be *Plasmodium falciparium*, 23.4% (170) were *Plasmodium malariae*, while 31 (4.27%) were *Plasmodium vivax*. No *Plasmodium ovale* was detected using the molecular techniques.To the best of our knowledge, this is the very first report in Nigeria where molecular approach beyond microscopy were used in evaluating the prevalence of species of *Plasmodium.* Amongst the species detected, *P. malariae*was the highest in the population. This is instructive in diagnosis of malaria as many health workers usually go for *P.falciparium* when assessing for these parasite using microscopy. Very similar to this report was done by Noland *et al*in 2014 in Abia State Nigeria, where 32.04 % of malaria infections were due to *P.malariae*(Noland *et al*., 2014).Though they relied completely on microscopy, the present work used molecular

approach. It appears from previous reports that southeast Nigeria has high incidence of *P.malariae.* Noland *et al*, in the work cited above also compared the prevalence of malaria in Abia and Plateau; he found out that 32.04 % and 1.4 % of malaria infections due to *P.malariae* were prevalent in Abia and Plateau respectively (Noland *et al*., 2014). Another report with low prevalent of *P.malariae* was done by Damen *et al*.(2015) in Jos, north central, Nigeria. It was reported that amongst healthy blood donors in North-Central Nigeria, parasitaemia due to *Plasmodium falciparum* was 98.0 % while 2.0 % was due to *P.malariae*(Damen *et al*., 2015). This is a pointer to the geographical distributions of these parasites. In southwest, Nigeria, malaria parasitaemia among blood donors were assessed by Oluwami *et al.* in 2015.It was reported in the said work that 85.7 % of *P.falciparum*and 14.3 % of *P.malariae* were detected in the blood samples analysed (Oluwami *et al*., 2015). Another work in Iwo community, southwestern Nigeria also revealed that the prevalence of *P.malariae* is high in southeast when compared with southwest. Out of the 733 malaria subjects examined, 93.3 % had *P.falciparum* while 6.7 % had *P.malariae* which is different from the report from southeast of Nigeria (Igbeneghu *et al*., 2012). Though, as mentioned earlier, all these reports depended solely on microscopy, the present work applied molecular technique in the analysis which also supported the data from previous works of this sort in the southeast Nigeria.

Early this year, Manego *et al.*, 2017 reported a high prevalence of *Plasmodium falciparum* (79 %) and low prevalence of *Plasmodium malaria* (23.2 %) in Tsamba-Magotsi, Ngounie Province, in Central African Gabon. Here, most of the *Plasmodium malaria* were as co-infection with *Plasmodium ovale* (Manego *et al*., 2017). Also, beyond Africa, Li *et al.* (2016) reported *P.malariae* as a minor parasite in China-Myanmar border area probably because of few number of them found there (4 of 561). In the molecular survey in that region, *P.vivax* was the predominant (327 of 561 cases) while *P.falciparum* and *P.malaria*were 161 and 4 of

561 respectively (Li *et al*., 2016). It appeared that the prevalence of *P.malariae* in Africa and beyond is low compared to the outcome of the present work.

Unexpectedly, around 4.27 % of *P.vivax* was detected in the samples analysed. These samples were further confirmed using real-time PCR machine. *P.vivax* were known to infect majorly Duffy positive individuals as goes the Duffy hypothesis(Miller *et al*., 1976). However, in the current time, there has been reports of *P.vivax* infecting Duffy negative individuals in malaria endemic regions of the world (Cavasini*et al*., 2007; Menard *et al*., 2010; Mendes *et al*., 2011; Ryan *et al*., 2006; Wurtz *et al*., 2011). This work confirmed three of the individuals with *P.vivax* infection as Duffy Negative individuals. These individuals were found to be negative of other species of *Plasmodium*. This negates the hypothesis that Duffy negative individuals are shielded from *P.vivax* infection. Though it is not clear how *P.vivax* goes into the erythrocyte of Duffy negative individuals, it could be that the parasite had undergone evolutionary changes to be able to do that. With other regions of Africa, we therefore hypothesize that a new *P.vivax* strain has appeared that does not depend on the Duffy antigen for entry into the erythrocyte.

Although three species of *Plasmodium* occurred sympatrically at Nnewi, southeast Nigeria, few of the infections were mixed infection. Following the result in Figure 47, 13.33 % samples had *P.falciparum* and *Plasmodium malariae*. 1.57 % samples had *P.falciparum* and *P.vivax* while 0.39 % sample had *P.falciparum*, *P.malariae* and *P.vivax* co-infections altogether. These were revealed by nested PCR, as microscopy could not provide any clear differential diagnosis. Microscopic diagnosis is the gold standard widely used for epidemiological studies. However, microscopy has both qualitative and quantitative limitations. When parasitaemia falls below 40 infected red blood cells per µl of blood, detection with microscopy becomes very difficult. Also, reproducibility of parasite count and species identification is irregular most times (Ohrt *et al*., 2002). Importantly, when one

parasite predominates in case of mixed infections, microscopy could detect only for the one with higher numbers of parasite (Genton *et al*., 2008). This is a challenge in routine microscopy and RDTs as rare malaria parasites and mixed infections are underestimated. This could lead to misidentification of malaria parasite resulting to delay parasite clearance, anaemia and drug resistance (de Roode *et al.*, 2004). Several works in the past had reported mixed infections in one blood samples. These ranged from Nigeria to other parts of the world. Umaru *et al*. in 2015 reported a case of mixed infection of *P.falciparum* and *P.ovale* in their work on Prevalence of Malaria in Patients Attending the General Hospital Makarfi, Kaduna-state, North – Western Nigeria (Umaru *et al*., 2015).In south west Nigeria, May *et al.* reported simultaneous infections with *P.falciparum*. *P.ovale* and *P.malariae* in Ibadan. They found 11.7 % of the population sampled with this level of co-infection (May *et al*. 1999). Beyond Nigeria, Manego *et al*. 2017 reported a high level of *P. falciparum* and *P. malariae* coinfections (23.2%) in an epidemiology survey they carried out in central African Gabon (Manego *et al*. 2017). Also, in Rattanakiri province, Cambodia Steenkeste *et al*.in 2010 reported 23 % prevalence of mixed infection with *P.falciparum* and *P.vivax* (Steenkeste *et al*., 2010). Recently, Abston *et al*. in 2017 reported a case of Midwestern returned traveller who was diagnosed of mixed –Species of malaria infection (Abston *et al*., 2017).Malaria mixed infections are normally underestimated. Chemotherapeutically, malaria control measures that aim at one species of *Plasmodium*may not achieve the desired result as cases of mixed infection is on the increase. This line of thought should also be extended to the laboratory for appropriate diagnosis of malaria infection.

### 5.1.4 Genetic Diversity of the *P.falciparum* strains

In Nigeria, very few studies investigated the genetic diversity and multiplicity of infection of *P.falciparum*. To our best of knowledge, this appears to be the first work to provide data on the genetic diversity and multiplicity of infection in Nnewi,South-eastern Nigeria. In the

present study, genetic diversity were assessed in three population cohorts comprising children, pregnant women and other adult population. In children population, MAD 20 family was the most predominant (29.16 %) within the MSP1 genewhile 3D7 family was the predominant (47.9 %) within the MSP2 gene. This is slightly different from the observations made in the other cohorts assessed. K1 (82.2 %) and 3D7 (60 %) were the most predominant in the women and mixed population cohorts respectively. These findings were slightly different with the study of Oyebola*et al.* in southwest Nigeria. They reported K1 as the most predominant within the MSP1 gene and FC27 family as the most predominant within the MSP2 gene(Oyebola*et al*., 2014). In another State in Southwest Nigeria, Ogun State precisely, Olasehinde *et al*., in 2012 had earlier reported higher prevalence of K1 within MSP1 family and 3D7 family within MSP2 gene (Olasehinde *et al*., 2012). This report is very similar to the report of the present study. In North Central Nigeria, Oyedeji *et al.* in 2013 assessed genetic diversity of *Plasmodium falciparum* isolates from naturally infected children using only merozoite surface protein-2 as molecular marker (Oyedeji *et al*., 2013). Their findings were different from the outcome of the present report, but similar to the study of Oyebola*et al.*(Oyebola*et al*.,2014). Beyond Nigeria, many works had been done to reveal the diversity of *P.falciparum* in some regions of the world. Contrary to the report of the present study, Saha*et al*. in 2016 reported high diversity of *P.falciparum* in West Bengal, India. They found R033 and FC27 to be dominant when compared to other families of MSP1 and MSP2 respectively in that region(Saha *et al*., 2016). The difference in the diversity report of Nigeria and India could be attributed to differences in the geographical zones. Some other studies from Thailand, Iran, Pakistan and Cameroon revealed that 3D7 was predominant over FC27 (Snounou *et al*., 1999; Basco *et al*., 2004; Zakeri *et al*., 2005; Ghanchi *et al*., 2010).

Average multiplicity of infection for MSP1, MSP2 and Glurp were 1.15, 2.40 and 1.4 respectively. This is slightly different from the report of study conducted in Lagos, Nigeria by

Oyebola*et al*. in 2014 (Oyebola*et al*.,2014). They found that multiplicity of infection for msp1 was between 1.39 and 1.50 for the localities sampled. Comparing the present study and that of Oyebola*et al*, Multiplicity of infection for msp-2 is higher in the south east Nigeria to south west Nigeria. This is in the opposite for msp-1 gene diversity. In North central Nigeria, Oyedeji *et al* in 2013 reported multiplicity of infection of 1.3 for msp-2 gene (Oyedeji *et al*., 2013). We can attempt to hypothesize from the foregone that *P.falciparum* diversity based on msp-2 gene is highest in south east Nigeria.

Presently, glurp and msp-3 are been explored as possible vaccine candidate for the development of malaria vaccine. This might only favour population with less diversity in the glurp gene. The present study showed that the multiplicity of infection for glurp gene is 1.4. This is different from the work of Kolawole *et al*. in 2016 where they reported multiplicity of infection for glurp gene as 2.05 in malaria patients attending Okeleke Health Centre, Okeleke, Ilorin, Kwara state, Nigeria (Kolawole *et al*., 2016). Also, still in southwest, Nigeria, Happi *et al*. in 2004 reported high diversity of glurp gene [2±1.38] in children population(Happi *et al*., 2004). It appears that *P. falciparum*glurp genes in the west are more diverse to that of the east.

Furthermore, the present study demonstrated that 81 % and 19 % of the isolates were monoclonal and polyclonal infections respectively for msp-1 gene, while 35.5 % and 64.5 % isolates harboured monoclonal and polyclonal infections respectively for msp-2 gene. This report suggest a very low complexity and moderate high complexity of *P.falciparum* msp-1 and msp-2 gene respectively in the area. Oyedeji *et al*. reported differently in their study using msp-2 gene. They reported high monoclonal infections (66 %) amongst children with severe malaria in North central, Nigeria (Oyedeji *et al*., 2013).

Heterozygosity was 0.49 – 0.59 for msp-1, 0.13 – 0.59 for msp-2 and 0.33 – 0.92 for glurp suggesting that the parasite in the population shows low to high heterozygosity, in line with the heterozygote range (0.51-0.65) reported in southeast Asia (Anderson *et al*., 2000). Anderson *et al* also reported that areas of declining endemicity, heterozygosity decreases with reduced transmission. (Anderson *et al.*, 2000).

### 5.1.5 Antimalarial Resistance Genes Single –Nucleotide Polymorphisms (SNPs)

Six genes, *pfmdr1, pfcrt, Pfkelch 13, Pfatpase, pfdhps* and *pfdhfr* were amplified and sequenced for single nucleotide polymorphisms studies. This is the first study in the southeast Nigeria on the prevalence of antimalarial resistance genes with such broad spectrum on asymptomatic persons.

Pfmdr1 gene was successfully sequenced in 82 samples amongst the *P.falciparum*isolates found in the study area. Frequency of N86Y was 91.46 % and 8.54 % for mutants and wild type respectively. Y184F had frequency of 67.07 % (wild type) and 52.78 % (mutants). Beyond the two known mutations mentioned, there are other point mutations found in other codon in the gene. Mutant *pfmdr1 N86Y* had been linked to pressure due to Amodiaquine in The Gambia (Duraisingh *et al.*, 1997), Kenya (Holmgren *et al.*, 2006)] and Nigeria (*Happi et al.*, 2006). Thus, high level N86Y in the present study could suggest that the parasite might be resistance to Amodiaquine in the study area. Studies have also shown that increased copy number of pfmdr1 gene increased the risk of failure after treatment with Mefloquine (Preechapornkul *et al.*, 2009).In Nigeria, some persons had attempted to assess this mutation. Happi *et al*. in 2003 reported the incidence of Y86 allele of pfmdr1 (40 %) in children with acute uncomplicated malaria (Happi *et al*., 2003). Ojurongbe *et al*. in 2010 reported 39 % and 35 % prevalence of Pfmdr1 in Lafia (North central Nigeria) and Oshogbo (southwest

Nigeria)respectively (Ojurongbe *et al*., 2010). 184F is predominance in the present work with some shown to be heterozygote (3.66 %).

55 *P.falciparum* isolates were sequenced for mutations in the *pfcrt* gene. In different parts of the world, chloroquine resistance is usually due to mutations at *pfcrt*. In this study, *pfcrt* gene mutation was observed in 94.54 % of the samples analysed. CVMNK haplotype was detected which is unusual in Nigeria. Nigeria is associated with CVIET haplotype. This outcome is a clear pointer that many health workers in Nigeria did not adhere to the withdrawal of Chloroquine in 2005.This could also be possibly attributed to drug pressure as a result of its continued use, cheap price and availability in many areas in the country (Alareqi*et al.,* 2016). In 2010, Bashrahil *et al*.reported chloroquine as the most prescribed antimalarial by general practitioner after the ACT policy (Bashrahil *et al.*, 2010). Continued prescription of the drug by the practitioners could be as a result of poor knowledge of the physicians about the WHO policy on ACT (Bin *et al*., 2013) Also, there may be persistent circulation of the marker due to drug selection pressure from amodiaquine, not necessarily from chloroquine. Additionally, the increase could also be as a result of cross-resistance to chloroquine (Fall *et al.,* 2011). Frequency of the *pfcrt* mutations in the study areas is higher than previous reports in southwest Nigeria (Happier*et al*., 2003; Ojurongbe *et al.,* 2007). The rise of the mutant allele in the present study is in contrast to its decline in some other African countries after withdrawal of chloroquine in Malawi (Kublin *et al.,* 2003; Laufer *et al.,* 2006), Tanzania (Alifrangis *et al*., 2009; Kamugisha *et al*., 2012; Malmberg *et al*., 2013; Mohammed *et al*., 2013), Kenya ( Mang'era *et al*., 2012; Mwai *et al*., 2009), Mozambique (Thomsen *et al*., 2013) and Ethiopia (Mekonnen *et al*., 2014).

Interests are gradually growing about the emergence of and spread of artemisinin-resistant *P.falciparum* in Southeast Asia (Phyto *et al*., 2012). Currently, Ashley *et al*. in 2014 reported a strong association between K13 –propeller mutations and delayed parasite clearance

(Ashley *et al*., 2014). This has not yet been confirmed outside Asia, though there had been report of K13- propeller mutations in sub-Saharan Africa (Kamau *et al*., 2014). The present study, to the best of our knowledge is the first report of K13 –propeller gene in Nigeria. 36 isolates were fully sequenced and analysed for K13 – propeller gene mutation. None of the isolates showed mutations in the already reported codons. Mutations observed are not common with K13-propeller gene. This finding is consistent with previous reports by Adeel *et al*., 2015.

Also, it has been established that *pfatpase*6 mutation is highly associated with raised artemeter IC50 in *Plasmodium falciparum* from French Guiana (Jambou *et al*. 2005). *Pfatpase*6 mutations were also assessed in the current work. Mutations were observed in codons 569K (8.33 %), 639D (4.17 %) and 1723V (4.17 %). Heterozygote allele was detected at codon 639 (4.17 %). None of the confirmed mutations associated with artemisinin resistance were detected.However, a study in Equatorial Guinea on artemisinin resistance associated polymorphisms reported a similar mutation at codons 1723 and 569 of *pfatapase*6 (Li *et al*., 2016). Polymorphisms evaluations of pfatapse6 in Africa is not common (Zatra *et al*., 2012). The current study first describes pfatapse6 polymorphisms in Nnewi, Southeast, Nigeria. Previous studies have also found high prevalence of N569K mutations in Zangiber (36 %),Tanzania (29%) (Dahlstrom *et al*., 2008) and Niger (17.2%) (Ibrahim *et al*., 2009).

Sulfadoxine – pyrimethamine was a promising drug against chloroquine-resistant strains of *Plasmodium falciparum* in Africa. However, this was compromised by emergence of SP resistant strains. The present work assessed the prevalence of DHFR and DHPS mutations in isolates recovered from pregnant women that were placed under IPT. In DHFR gene, mutations were observed at codons 511I (57.14 %), 59R (71.43 %) and 108N (71.43 %), while in DHPS gene, mutations were observed at codons 431V (40 %), 436A (50%), 581G (20 %) and 613S (50 %).Similar report occurred in a work by Ojurongbe *et al*., 2011. They

assessed the frequency of DHFR mutations associated with drug resistance among *P. falciparum* isolates from pregnant women in Ilorin, Nigeria (Ojurongbe *et al*.2011). Though only DHFR gene was assessed in their report, but similar mutations with the present work were observed (codons 59 and 108). Currently, Oguike *et al*. 2016 reported similar mutations when they assessed the frequency of SP resistance across Southsouth, Southeast and Northeast regions of Nigeria. In line with the present study, they reported high prevalence of 431V (novel dhps mutations) amongst other mutations reported. The efficacy of IPTp-SP had been compromised by these mutations. WHO has recommended that before implementation of IPTp-SP in any region with moderate to high malaria transmission, the prevalence of K540E and A581G should be assessed. IPTp-SP should be used in regions with a prevalence rate K540E less than 50 % and A581G less than 10 % (WHO, 2013).

*Plasmodium falciparum* Circumcporozoite gene (CSP) was successfully sequenced in 33 isolates from the region. The central repeat region was identified and mapped against reference 3D7. The variations observed was high which might also affect the immunogenicity of the CSP region when used as a vaccine component. This is important as PfCSP from 3D7 was used in the design of RTS,S vaccine. From the variations observed, there is doubt that RTS,S vaccine would provide good immunogenic protection as it were. This is further confirmed from the Phylogenetic analysis outcome (Figure 57) with few clusters of the sequences.

### 5.2 Inferences, Conclusion and Summary

The present study is a molecular survey on the epidemiology of *Plasmodium falciparum* amongst asymptomatic carriers in Nnewi, Southeast Nigeria. Preliminary data on the usage of preventive tools against mosquito bites showed that majority of the persons in the populations cared less. Also, preliminary data shows that levels of education had no impact on the use of

mosquito nets in the population. Calculated mean parasite densities were not too high as the population under study were asymptomatic carriers. Levels of parasite densities in an individual is not a function of age, but genetics of the person concern. Multivariate analysis showed that use of either mosquito net or repellent has no direct effect on the parasite density. Molecular epidemiology using PCR showed high prevalence of *Plasmodium malaria* in the area under study. The study also is the first report of *Plasmodium vivax* in Duffy negative individuals. Genetic diversity of the resultant *P.falciparum* in the population showed high diversity of 3D7 family in msp-2 gene. Also, the study revealed that the predominant family in msp-1 gene is MAD 20. Sequencing also showed further variations in the families with the same band size in gel.

It was also observed that antimalarial resistance genes are on the increase in the region studied. Chloroquine resistance marker still persists in the population.

### 5.3 Recommendation

Regular large scale surveillance of molecular markers of resistance to the antimalarial components of the current antimalarial treatment policy is recommended. Meanwhile, educational programs on the effects of bad treatment – seeking behaviours and antimalarial drug misuse on the emergence and spread of drug resistance should be launched for physicians, pharmacists and general population in endemic areas.

### 5.4 Contribution to Knowledge

-The present study had provided a baseline data on the *Plasmodium falciparum* antigenic markers circulating in Nnewi, Southeast Nigeria

- The study, for the first time had described the emergence of *Plasmodium vivax* in Duffy Negative individuals in Nigeria

- Against the previous believe that *Plasmodium falciparum* is the highest circulating species, the present study had shown *P. malariae* as the most predominant in the region.

- The study has also shown that *Pfcrt* marker is still in circulation in the region despite WHO withdrawal of the same in 2005.

- It's also known from the study that artemisinin resistance gene is not yet in circulation in the region studied.

## REFERENCES


Abilio, A. P., Kleinschmidt, I., Rehman, A. M., Cuamba, N., Ramdeen, V., Mthembu, D. S., Coetzer, S., Maharaj, R., Wilding, C, S., Steven, A., Coleman, M., Hemingwway, J. and Coleman, M.(2011). The emergence of insecticide resistance in central Mozambique and potential threat to the successful indoor residual spraying malaria control programme. *Malaria Journal*, *10*(1), 110. https://doi.org/10.1186/1475-2875-10-110

Abston, E., Rooney, S., Johnson, M. and Haleem, A. (2017). Mixed-Species Malaria Infection in Midwestern Returned Traveler. *The American Journal of the Medical Sciences.* 2017;353:879.

Adeel, A.A., Saeed, N.A., Aljasari, A., Almohager, A.M., Galab, M.H., AlMahdi, A.,Mahammed, M.H., AlDarsi, M., Salaeah, Y.A., Atta, H., Zamani, G., Warsame, M., Barrette, A., Mohammady, H. E. and Nada, R. A.(2015). High efficacy of twoartemisinin-based combinations: artesunate + sulfadoxine-pyrimethamine andartemether-lumefantrine for *P.falciparum* malaria in Yemen. *Malar. J.* 14, 449.

Adebayo, N. S., Oladele, H. A., Olalekan, A. W., Akintunde, A. R., Microbiology, M., Raimi, O., … Nneoma, C. A. (2012). Malaria and Anaemia in Pregnant and Non-Pregnant Women of Child-Bearing Age at the University Hospital, Kumasi, Ghana. *Asian Pacific Journal of Reproduction*, *3*(3), 415–422. https://doi.org/10.2147/PPA.S61448

Afoakwah, R., Boampong, J. N. and Acheampong, D. O. (2011). Polymorphisms in Plasmodium falciparum Adenosine Triphosphatase 6 (PfATPase6) gene and their significance in finding the genetic marker for Artemisinin resistance. *European Journal of Experimental Biology*, *1*(3), 7–13.

Agathe, B., Bécaye, F., Marylin, M., Khalifa A.W. Mansour, F., Aminata, N., Khadidiatou B. F., Pierre, D., Nicolas, B.,Bakary, D., Yaya, D., Boubacar, W. and Bruno, P. (2016). Prevalence of anti‑malarial resistance genes in Dakar, Senegal from 2013 to 2014. *Malar J* 15:347 DOI 10.1186/s12936-016-1379-2.

Ahmedou Salem, M. S. O., Ndiaye, M., OuldAbdallahi, M., Lekweiry, K. M., Bogreau, H., Konaté, L., Faye, B., Gaye, O., Ousmane F. and Mohamed Salem O Boukhary, A. O. (2014). Polymorphism of the merozoite surface protein-1 block 2 region in Plasmodium falciparum isolates from Mauritania. *Malaria Journal*, *13*, 26.https://doi.org/10.1186/1475-2875-13-26

Alareqi, L. M. Q., Mahdy, M. A. K., Lau, Y. L., Fong, M. Y., Abdul-Ghani, R., and Mahmud, R. (2016). Molecular markers associated with resistance to commonly used antimalarial drugs among *Plasmodium falciparum* isolates from a malaria-endemic area in Taiz governorate-Yemen during the transmission season. *Acta Tropica*, *162*, 174–179. https://doi.org/10.1016/j.actatropica.2016.06.016

Alifrangis, M., Lusingu, J.P., Mmbando, B., Dalgaard, M.B., Vestergaard, L.S.,Ishengoma, D., Khalil, I.F., Theander, T.G., Lemnge, M.M., and Bygbjerg, I. C. (2009).Five-year surveillance of molecular markers of *Plasmodium falciparum*antimalarial drug resistance in Korogwe District, Tanzania: accumulation ofthe 581 G mutation in the *P. falciparum* dihydropteroate synthase gene. *Am. J.Trop. Med. Hyg*. 80, 523–527.

Ambroise-Thomas, P. (2012). The tragedy caused by fake antimalarial drugs. *Mediterranean journal of hematology and infectious diseases*, *4*(1), 2012027.

Anderson, T.J.C., Bernhard, H., Williams, J.T., Estrada-Franco, J.G., Richardson, L., Mollinedo, R., Bockarie, M., Mokili, J., Maharakurwa, S., French, N., Whitworth, J., Velez, I.D., Brockman, A.H., Nosten, F., Ferreira, M.U., and Day, K.P. (2000). Microsatellites reveal a spectrum of population structure in the malaria parasite *Plasmodium falciparum.Mol. Biol. Evol.* 17, 1467–1482.

Andre, F. E., Booy, R., Bock, H. L., Clemens, J., Datta, S. K., John, T. J., Lee, B. W., Lolekha, S., Peltola, H., Ruff, T. A., Santosham, M., Schmitt,H. J. (2008). Vaccination greatly reduces disease, disability, death and inequity worldwide. *Bulletin of the World Health Organization*, *86*(2), 140-146.

Ankomah, A., Adebayo, S. B., Arogundade, E. D., Anyanti, J., Nwokolo, E., Ladipo, O. and Meremikwu, M. M. (2012). Determinants of insecticide-treated net ownership and utilization among pregnant women in Nigeria. *BMC Public Health*; 12:105.

Antony, H. A., and Parija, S. C. (2016). Antimalarial drug resistance: An overview. *Tropical Parasitology*, *6*(1), 30–41. https://doi.org/10.4103/2229-5070.175081

Antosiewicz, J., McCammon, J. A., and Gilson, M. K. (1994). Prediction of Ph-dependent Properties of Proteins. *Journal of Molecular Biology*, *238*(3), 415–436. https://doi.org/10.1006/jmbi.1994.1301

Apinjoh, T. O., Anchang-Kimbi, J. K., Mugri, R. N., Tangoh, D. A., Nyingchu, R. V., Chi, H. F.,Tata R. B., Njumkeng,C., Njua-Yafi, C. andAchidiE. A. (2015). The Effect of Insecticide Treated Nets (ITNs) on *Plasmodium falciparum* Infection in Rural and Semi-Urban Communities in the South West Region of Cameroon. *PLoS One* [Internet]. [cited 2017 Jan 22];10. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4340618/

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A. C., Khim, N., KimS., Duru, V., Bouchier, C., Ma, L., Lim, P., Leang, R., Duong, S., Sreng, S., Suon, S., Chuor, M. C., Bout, D. M., Ménard, S.,Rogers, W. O., Genton, B., Fandeur, T., Miotto, O., Ringwald, P., Bras, J. L., Berry, A., Barale, J., Fairhurst, R. M., Benoit-Vical, F., Puijalon O. M. and Ménard D. (2014). A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. *Nature*, *505*(7481), 50–55. https://doi.org/10.1038/nature12876

Arnou, B., Montigny, C., Morth, J. P., Nissen, P., Jaxel, C., Møller, J. V., and Maire, M. le. (2011). The Plasmodium falciparum Ca(2+)-ATPase PfATP6: insensitive to

artemisinin, but a potential drug target. *Biochemical Society Transactions*, *39*(3), 823–831. https://doi.org/10.1042/BST0390823

Ashley, E.A., Dhorda, M., Fairhurst, R.M., Amaratunga, C., Lim, P., Suon, S., Sreng, S.,Anderson, J.M., Mao, S., and Sam, B.(2014). Spread of artemisinin resistance in*Plasmodium falciparum* malaria. *N. Engl. J. Med*. 371, 411–423.

Ayieko, C., Ogola, B. S., Ochola, L., Ngwena, G. A. M., Ayodo, G., Hodges, J. S. NolandG. S. and John C. C. (2017). Interferon- $\gamma$ responses to *Plasmodium falciparum* vaccine candidate antigens decrease in the absence of malaria transmission. *PeerJ*, *5*, e2855. https://doi.org/10.7717/peerj.2855

Balikagala, B., Mita, T., Ikeda, M., Sakurai, M., Yatsushiro, S., Takahashi, N.Tachibana, S., Auma, M., Ntege, E. H., Ito, D., Takashima, E., Palacpac, N. M. Q., Egwang, T. G., Onen, J. O., Kataoka, M., Kimura, E., Horii T. and Tsuboi T., (2017). Absence of in vivo selection for K13 mutations after artemether–lumefantrine treatment in Uganda. *Malaria Journal*, *16*(1), 23. https://doi.org/10.1186/s12936-016-1663-1

Ballou, W. R., Arevalo-Herrera, M., Carucci, D., Richie, T. L., Corradin, G., Diggs, C.,Druilhe, P., Giersing, B. K., Saul, A., Heppner, D. G., Kester, K. E., Lanar, D. E., Lyon, J., Hill, A. V. S., Pan, W. and Cohen,J. D. (2004). Update on the clinical development of candidate malaria vaccines. *The American Journal of Tropical Medicine and Hygiene*, *71*(2 Suppl), 239–247.

Bannister, L, H., Mitchell, G. H., Butcher, G. A., Dennis, E. D., Cohen, S (1986). Structure and development of the surface coat of erythrocytic merozoites of *Plasmodium knowlesi*. *Cell and Tissue Research*, *245*,(2), 281-290.

Barrera, S. M., Perez, M. A., Knudson, A., Nicholls, R. S., and Guerra, A. P. (2010). Genotypic survey of *Plasmodium falciparum* based on the msp1, msp2 and glurp genes by multiplex PCR. *Biomedica*, *30*(4), 530–538. https://doi.org/S0120-41572010000400010 [pii]

Basco, L.K., Tahar, R., and Escalante, A. (2004). Molecular epidemiology of malaria in Cameroon. XVIII. Polymorphisms of the *Plasmodium falciparum* merozoite surface antigen-2 gene in isolates from symptomatic patients. *Am.J.Trop. Med. Hyg*. 70, 238–244.

Bashrahil, K., Bingouth, A., and Baruzaig, A. (2010). Antimalarial drugs: Availability and mode of prescribing in Mukalla, Yemen. East. Mediterr. *Health J.* 16, 146–150.

Baume, C. A., Reithinger, R., and Woldehanna, S.(2009). Factors associated with use and non-use of mosquito nets owned in Oromia and Amhara Regional States, Ethiopia. *Malaria Journal*, 8:264.

Bezirtzoglou, C., Dekas, K. and Charvalos, E. (2011). Climate changes, environment and infection: facts, scenarios and growing awareness from the public health community within Europe. *Anaerobe*, *17*(6), 337-340.

Bin Ghouth, A.S.(2013). Availability and prescription practice of anti-malaria drugsin the private health sector in Yemen. *J. Infect. Dev. Ctries.* 7, 404–412.

Boccolini, D., Toma, L., Luca, M. Di, Severini, F., Cocchi, M., Bella, A.Massa, A., Mancini F. B., Bongiorno G., Angeli, L., Pontuale, G., Raffaelli, I., Fausto, A. M., Tamburro, A. and Romi,R. (2012). Impact of Environmental Changes and Human-Related Factors on the Potential Malaria Vector, Anopheles labranchiae (Diptera: Culicidae), in Maremma, Central Italy. *Journal of Medical Entomology*, *49*(4), 833–842. https://doi.org/10.1603/ME11252

Boland, P. B. (2001). Drug resistant malaria. *World Health Organisation, Geneva. WHO/CDS/CSR/DRS*.

Borre, M. B., Dziegiel, M., Høgh, B., Petersen, E., Rieneck, K., Riley, E.,Meis, J. F., Aikawa, M., Namura, K., Harada, M., Wind, A., Jakobsen, P. H., Cowland, J., Jepsen, S., Axelsen, N. H. and Vuust, J. (1991). Primary structure and localization of a conserved immunogenic *Plasmodium falciparum* glutamate rich protein (GLURP) expressed in both the preerythrocytic and erythrocytic stages of the vertebrate life cycle. *Molecular and Biochemical Parasitology*, *49*(1), 119–31. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1775153

Boyle, M. J., Wilson, D. W. and Beeson, J. G. (2013). New approaches to studying Plasmodium falciparum merozoite invasion and insights into invasion biology. *nternational Journal for Parasitology*, *43*(1), 1–10. https://doi.org/10.1016/j.ijpara.2012.11.002

Bray, P. G., Martin, R. E., Tilley, L., Ward, S. A., Kirk, K. and Fidock, D. A. (2005). Defining the role of PfCRT in Plasmodium falciparum chloroquine resistance. *Molecular Microbiology*, *56*(2), 323–333. https://doi.org/10.1111/j.1365-2958.2005.04556.x

Bruder, J. T., Angov, E., Limbach, K. J. and Richie, T. L. (2010). Molecular vaccines for malaria. *Human Vaccines*, *6*(1), 54–77. https://doi.org/10.4161/hv.6.1.10463

Burrows, J. (2015). Malaria runs rings round artemisinin. *Nature*, *520*, 628–630. https://doi.org/10.1038/nature14387

Callaghan, P. S., Hassett, M. R. and Roepe, P. D. (2015). Functional Comparison of 45 Naturally Occurring Isoforms of the Plasmodium falciparum Chloroquine Resistance Transporter (PfCRT). *Biochemistry*, *54*(32), 5083–5094. https://doi.org/10.1021/acs.biochem.5b00412

Callaghan, P. S., Siriwardana, A., Hassett, M. R. and Roepe, P. D. (2016). Plasmodium falciparum chloroquine resistance transporter ( PfCRT ) isoforms PH1 and PH2 perturb vacuolar physiology. *Malaria Journal*, 1–13. https://doi.org/10.1186/s12936-016-1238-1

Carvalho, L. J. M., Daniel-Ribeiro, C. T. and Goto, H. (2002). Malaria vaccine: Candidate antigens, mechanisms, constraints and prospects. *Scandinavian Journal of Immunology*, *56*(4), 327–343. https://doi.org/10.1046/j.1365-3083.2002.01160

Cavasini, C. E., Mattos, L. C., Couto, A. A., Bonini-Domingos, C. R., Valencia, H. S., de Souza Neiras, W. C., Alves, T, R., Rossit, B. R. A., Castilho, L. and Machado, R. L. D. (2007). *Plasmodium vivax* infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception? T*rans R Soc Trop Med Hyg*. 101:1042–4.

CDC, (2013). http://www.cdc.gov/malaria/about/biology/parasites.html, accessed on 7th April 2016

CDC, (2016). Malaria - Malaria Worldwide - Impact of Malaria. Retrieved February 16, 2017, from https://www.cdc.gov/malaria/malaria_worldwide/impact.html

Chandramohanadas, R., Russell, B., Liew, K., Yau, Y. H., Chong, A., Liu, M., Gunalan K.,Raman, R., Renia, L., Nosten, F., Shochat, G. S., Dao, M., Sasisekharan, R., Suresh, S., Preiser, P., (2014). Small molecule targeting malaria Merozoite surface protein-1 (MSP-1) prevents host invasion of divergent Plasmodial species. *Journal of Infectious Diseases*, *210*(10), 1616–1626. https://doi.org/10.1093/infdis/jiu296

Chaturvedi, N., Bharti, P. K., Tiwari, A. and Singh, N. (2016). Strategies & recent development of transmission-blocking vaccines against *Plasmodium falciparum*. *Indian Journal of Medical Research*, *143*, 696–711. https://doi.org/10.4103/0971-5916.191927

Cheriyedath, S. (2017). History of Polymerase Chain Reaction ( PCR ), 1–5. Retrieved from http://www.news-medical.net/life-sciences/History-of-Polymerase-Chain-Reaction-(PCR).aspx

Chitarra, V., Holm, I., Bentley, G. A., Pêtres, S. and Longacre, S. (1999). The crystal structure of C-terminal merozoite surface protein 1 at 1.8 Å resolution, a highly protective malaria vaccine candidate. *Molecular Cell*, *3*(4), 457–464. https://doi.org/10.1016/S1097-2765(00)80473-6

Chiu, C. Y., White, M. T., Healer, J., Thompson, J. K., Siba, P. M., Mueller, I., Cowman, A. F. and HansenD. S. (2016). Different Regions of *Plasmodium falciparum* Erythrocyte-Binding Antigen 175 Induce Antibody Responses to Infection of Varied Efficacy. *Journal of Infectious Diseases*, 214, 96–104.

Cowman, A. F., Baldi, D. L., Duraisingh, M., Healer, J., Mills, K. E., O'Donnell, R. A, Thompson, J., Triglia, T., Wickham, M. E. and Crabb, B. S. (2002). Functional analysis of *Plasmodium falciparum* merozoite antigens: implications for erythrocyte invasion and vaccine development. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *357*(1417), 25–33. https://doi.org/10.1098/rstb.2001.1010

Cowman, A. F., Berry, D. and Baum, J. (2012). The cellular and molecular basis for malaria parasite invasion of the human red blood cell. *Journal of Cell Biology*, *198*(6), 961–971. https://doi.org/10.1083/jcb.201206112

Cui, L., Wang, Z., Jiang, H., Parker, D., Wang, H., Su, X.Z. and Cui, L. (2012). Lack of association of the S769N mutation in Plasmodium falciparum SERCA (PfATP6) with

resistance to artemisinins. *Antimicrobial Agents and Chemotherapy*, *56*(5), 2546–52. https://doi.org/10.1128/AAC.05943-11

Damen, J. G, Barnabas, O., Damulak,D., Ntuhun, B. D., Lugos, M. D. and Nyary, B. (2015). Malaria parasitemia in apparently healthy blood donors in north-central Nigeria. *Lab Med.*,46:42–6.

Das, D., Tripura, R., Phyo, A. P., Lwin, K. M., Tarning, J., Lee, S. J., Hanpithakpong, W., Stepniewska, K., Menard, D., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Yi, P., Day, N. P. J., Lindegardh, N., Socheat, D., Nguon, C., White, N. J., Nosten, F. and Dondorp, A. M. (2013). Effect of High-Dose or Split-Dose Artesunate on Parasite Clearance in Artemisinin-Resistant Falciparum Malaria. *Clinical Infectious Diseases*, *56*(5), e48–e58. https://doi.org/10.1093/cid/cis958

de Roode J. C., Culleton, R., Bell, A. S. and Read, A. F. (2004). Competitive release of drug resistance following drug treatment of mixed Plasmodium chabaudi infections. *Malar J.*;3:33

Doolan, D. L., Hoffman, S. L., Southwood, S., Wentworth, P. A., Sidney, J., Chesnut, R. W. Keogh, E., Appella, E., Nutman, T. B., Lal, A., Gordon, D. M., Oloo, A. and Sette, A. (1997). Degenerate cytotoxic T cell epitopes from *P. falciparum* restricted by multiple HLA-A and HLA-B supertype alleles. *Immunity*, *7*(1), 97–112. https://doi.org/10.1016/S1074-7613(00)80513-0

Draper, S. J., Angov, E., Horii, T., Miller, L. H., Srinivasan, P., Theisen, M. and Biswas, S. (2015). Recent advances in recombinant protein-based malaria vaccines. *Vaccine*, *33*(52), 7433–7443. https://doi.org/10.1016/j.vaccine.2015.09.093

Duraisingh, M. T, Drakeley, C. J., Muller, O., Bailey, R., Snounou, G., Targett, G. A., Greenwood B. M. and Warhurst, D. C. (1997) Evidence for selection for the tyrosine-86 allele of the pfmdr 1 gene of *Plasmodium falciparum* by chloroquine and amodiaquine. *Parasitology* 114 (Pt 3): 205–211.

Duraisingh, M. T. and Cowman, A. F. (2005). Contribution of the pfmdr1 gene to antimalarial drug-resistance. *Acta Tropica*, *94*(3), 181–190. https://doi.org/10.1016/j.actatropica.2005.04.008

Duru, K. C. and Thomas, B. N. (2014). Genetic Diversity and Allelic Frequency of Glutamate-Rich Protein (GLURP) in *Plasmodium falciparum* Isolates from Sub-Saharan Africa. *Microbiology Insights*, *7*, 35–39. https://doi.org/10.4137/MBI.S13639.RECEIVED

Edelu, B., Ikefuna, A., Emodi, J. and Adimora, G. (2010). Awareness and use of insecticide-treated bed nets among children attending outpatient clinic at UNTH, Enugu - the need for an effective mobilization process. *African Health Sciences*; 10:117–9.

Epstein, J. E., Giersing, B., Mullen, G., Moorthy, V. and Richie, T. L. (2007). Malaria vaccines: are we getting closer? *Curr Opin Mol Ther*; *9* : 12-24.

Fall, B., Diawara, S., Sow, K., Baret, E., Diatta, B., Fall, K. B, Mbaye,P. S., Fall, F., Diémé, Y., Rogier, C., Wade, B., Bercion R. and Pradines, B. (2011). Ex vivo susceptibility of Plasmodium isolates from Dakar, Senegal, to seven standard antimalarial drugs. *Malar J.*;10:310

Felger, I., Irion, A., Steiger, S and Beck, H. P. (1999) Genotypes of merozoite surface protein 2 of Plasmodium falciparum in Tanzania.*Transactions of the Royal Society of Tropical Medicine and Hygiene*, , 93 Suppl 1:3-9

Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M. T., Ursos, L. M. B., Sidhu, A. S., Naudé, B., Deitsch, K. W., Su, X., Wootton, J. C., Roepe, P. D., and Wellems, T. E.(2000). Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular Cell*, *6*(4), 861–71. https://doi.org/10.1016/S1097-2765(05)00077-8

Flori. L., Kumulungui, B., Aucan, C., Esnault, C., Traoré, A. S., Fumoux, F.and Rihet, P. (2003). Linkage and association between *Plasmodium falciparum* blood infection levels and chromosome 5q31–q33. *Genes Immun.*, 4:265–8.

Foote, S. J. and Cowman, A. F. (1994). The mode of action and the mechanism of resistance to antimalarial drugs. *Acta Tropica*, *56*(2–3), 157–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8203302

Fuehrer, H. P., Stadler, M. T., Buczolich, K., Bloeschl, I., and Noedl, H.(2012). Two techniques to simultaneously identify *Plasmodium ovalecurtisi* and *P. ovale wallikeri* using the small subunit rRNA gene. *J Clin Microbiol*.;50:4100–4102.

Gamo, F. J. (2014). Antimalarial drug resistance: new treatments options for Plasmodium. *Drug Discovery Today: Technologies*, *11*, 81–88. https://doi.org/10.1016/j.ddtec.2014.03.002

Garcia, L. S. (2010) Malaria. *Clin Lab Med*; 30(1): 93-129.

Genton, B., D'Acremont, V., Rare, L., Baea, K., Reeder, J. C, and Alpers, M. P. (2008). *Plasmodium vivax* and mixed infections are associated with severe malaria in children: a prospective cohort study from Papua New Guinea. *PLoS Med*; 5:e127

Ghanchi, N.K., Martensson, A., Ursing, J., Jafri, S., Bereczky, S., Hussain, R. and Beg, M. A.(2010). Genetic diversity among *Plasmodium falciparum* field isolates in Pakistan measured with PCR genotyping of the merozoite surface protein 1 and 2. *Malar. J.* 9, 1.

Githinji, S., Herbst, S., Kistemann, T. and Noor, A.M. (2010). Mosquito nets in a rural area of Western Kenya: ownership, use and quality. *Malaria Journal*. 9:250.

Goswami, D., Dhiman, S., Rabha, B., Kumar, D., Baruah, I., Sharma, D. K. and Veer, V. (2014). Pfcrt mutant haplotypes may not correspond with chloroquine resistance. *The Journal of Infection in Developing Countries*, *8*(6), 768–773. https://doi.org/10.3855/jidc.3398

Happi, C. T., Gbotosho, G. O., Folarin, O. A., Bolaji, O. M., Sowunmi, A., Kyle, D. E,Milhous, W.,Wirth D. F. and OduolaA. M. J. (2006) Association between mutations in *Plasmodium falciparum* chloroquine resistance transporter and *P. falciparum* multidrug resistance 1 genes and in vivo amodiaquine resistance in *P. falciparum* malaria-infected children in Nigeria. *The American Journal of Tropical Medicine and Hygiene* 75: 155–161

Happi, C. T., Gbotosho, G. O., Sowunmi, A., Falade, C. O., Akinboye, D. O., Gerena, L. and Oduola, A. M. J. (2004). Molecular analysis of *Plasmodium falciparum* recrudescent malaria infections in children treated with chloroquine in Nigeria. *The American Journal of Tropical Medicine and Hygiene*, 70(1), 20–26.

Happi, T. C., Thomas, S. M., Gbotosho, G. O., Falade, C. O., Akinboye, D. O., Gerena, L. and Oduola, A. M. J. (2003). Point mutations in the pfcrt and pfmdr-1 genes of *Plasmodium falciparum* and clinical response to chloroquine, among malaria patients from Nigeria. *Annals of Tropical Medicine and Parasitology*, *97*(5), 439–451. https://doi.org/10.1179/000349803235002489

Hemming-Schroeder, E., and Lo, E. (2017). Evidence of a hard selective sweep for artemisinin resistant Plasmodium falciparum. *The Lancet Infectious Diseases*, *3099*(17), 1–2. https://doi.org/10.1016/S1473-3099(17)30056-7

Hoffman, S. L., Vekemans, J., Richie, T. L. and Duffy, P. E. (2015). The March Toward Malaria Vaccines. *American Journal of Preventive Medicine*, *49*(6), S319–S333. https://doi.org/10.1016/j.amepre.2015.09.011

Holder, A. A., Blackman, M. J., Burghaus, P. A., Chappel, J. A., Ling, I. T., McCallum-Deighton, N. and Shai, S. (1992). A malaria merozoite surface protein (MSP1)-structure, processing and function. *Memorias Do Instituto Oswaldo Cruz*. https://doi.org/10.1590/S0074-02761992000700004

Holmgren, G., Gil, J. P., Ferreira, P. M., Veiga, M. I., Obonyo, C. O.and Bjorkman, A. (2006) Amodiaquine resistant *Plasmodium falciparum* malaria in vivo is associated with selection of pfcrt 76T and pfmdr1 86Y. Infection, genetics and evolution: *Journal of molecular epidemiology and evolutionary genetics in infectious diseases*(6): 309–314

Hughes, A. L. (1991). Circumsporozoite protein genes of malaria parasites *(Plasmodium* spp.): Evidence for positive selection on immunogenic regions. *Genetics*, *127*(2), 345–353. https://doi.org/10.1016/0092-8674(85)90093-5

Humphreys, G. S., Merinopoulos, I., Ahmed, J., Whitty, C. J., Mutabingwa, T. K., Sutherland, C. J. and Hallett, R. L. (2007) Amodiaquine and artemether-lumefantrine select distinct alleles of the *Plasmodium falciparum* mdr1 gene in Tanzanian children treated for uncomplicated malaria. *Antimicrob Agents Chemother*, 51:991–997

Hunt, R. H., Fuseini, G., Knowles, S., Stiles-Ocran, J., Verster, R., Kaiser, M. L.,Choi, K.S., Koekemoer L. L. and Coetzee,M. (2011). Insecticide resistance in malaria vector mosquitoes at four localities in Ghana, West Africa. *Parasites {&} Vectors*, *4*(1), 107. https://doi.org/10.1186/1756-3305-4-107

Hutchison, C. A. (2007). DNA sequencing: Bench to bedside and beyond. *Nucleic Acids Research*, *35*(18), 6227–6237. https://doi.org/10.1093/nar/gkm688

Hyde, J. E. (2005). Exploring the folate pathway in Plasmodium falciparum. *Acta Tropica*, *94*(3), 191–206. https://doi.org/10.1016/j.actatropica.2005.04.002

Hyde, J. E. (2007). Drug-resistant malaria−an insight. *FEBS Journal*, *274*(18), 4688–4698. https://doi.org/10.1111/j.1742-4658.2007.05999.x

Ibraheem, Z. O., Abd Majid, R., Noor, S. M., Sedik, H. M., and Basir, R. (2014). Role of Different Pf crt and Pf mdr-1 Mutations in Conferring Resistance to Antimalaria Drugs in Plasmodium falciparum. *Malaria Research and Treatment*, *2014*, 1–17. https://doi.org/10.1155/2014/950424

Igbeneghu, C. and Odaibo, A. B. (2012). Plasmodium Species among the Inhabitants of Iwo Community, Southwestern Nigeria. *Am-Euras. J.Sci.Res*; 7 (3): 118 – 122.

Imwong, M., Dondorp, A. M., Nosten, F., Yi, P., Mungthin, M., Hanchana, S.Das, D., Phyo, A. P., Lwin, K. M., Pukrittayakamee, S., Lee, S. J., Saisung, S., Koecharoen, K., Nguon, C., Day, N. P. J., Socheat, D. and White, N. J. (2010). Exploring the contribution of candidate genes to artemisinin resistance in plasmodium falciparum. *Antimicrobial Agents and Chemotherapy*, *54*(7), 2886–2892. https://doi.org/10.1128/AAC.00032-10

Imwong, M., Suwannasin, K., Kunasol, C., Sutawong, K., Mayxay, M., Rekol, H.Smithuis, F. M., Hlaing, M., Tun, K. M., Van Der Pluijm, R. W., Tripura, R., Miotto, O., Menard, D., Dhorda, M., Day, N.P. J., White, N. J. and Dondorp, A. M. (2017). The spread of artemisinin-resistant Plasmodium falciparum in the Greater Mekong Subregion: a molecular epidemiology observational study. *The Lancet Infectious Diseases*, *3099*(17), 13–19. https://doi.org/10.1016/S1473-3099(17)30048-8

Integrated DNA Technologies. (2011). *DNA Sequencing*.

Iwuafor, A. A., Egwuatu, C. C., Nnachi, A. U., Ita, I, O., Ogban, G. I., Akujobi, C. N. and Egwuatu, O. T. (2016) Malaria Parasitaemia and the use of insecticide-treated nets (INTs) for malaria control amongst under-5 year old children in Calabar, Nigeria. *BMC Infect Dis* [Internet]. [cited 2017 Jan 22];16. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4832572/

Japrung, D., Chusacultanachai, S., Yuvaniyama, J., Wilairat, P. and Yuthavong, Y. (2005). A simple dual selection for functionally active mutants of Plasmodium falciparum

dihydrofolate reductase with improved solubility. *Protein Engineering Design and Selection*, *18*(10), 457–464. https://doi.org/10.1093/protein/gzi044

John, C. C., Tande, A. J., Moormann, A. M., Sumba, P. O., Lanar, D. E., Min, X. M. and Kazura, J. W. (2008). Antibodies to pre-erythrocytic *Plasmodium falciparum* antigens and risk of clinical malaria in Kenyan children. *The Journal of Infectious Diseases*, *197*(4), 519–26. https://doi.org/10.1086/526787

Jones, M. K. and Good M.F. (2006). Malaria parasites up close. *Nat. Med*. 12, 170-171

Jones, R. M., Chichester, J. A., Mett, V., Jaje, J., Tottey, S., Manceva, S., Casta, L. J., Gibbs, K. S., Musiychuk, K., Shamloul, M., Norikane, J., Mett, V., Streatfield, S. J., Vegte-Bolmer, M. V., Roeffen, W., Sauerwein, R. W. and Yusibov, V. (2013). A plant-produced Pfs25 VLP malaria vaccine candidate induces persistent transmission blocking antibodies against Plasmodium falciparum in immunized mice. *PloS one*, *8*(11), e79538

Kamau, E., Campino, S., Amenga-Etego, L., Drury, E., Ishengoma, D., Johnson, K.,Mumba, D., Kekre, M., William, Y. and Mead, D. (2014). K13-propellerpolymorphisms in *Plasmodium falciparum* parasites from sub-Saharan Africa. *J.Infect. Dis*. 211, 1352–1355

Kamugisha, E., Bujila, I., Lahdo, M., Pello-Esso, S., Minde, M., Kongola, G.,Naiwumbwe, H., Kiwuwa, S., Kaddumukasa, M., and Kironde, F. (2012). Large differences in prevalence of Pfcrt and Pfmdr1 mutations between Mwanza,Tanzania and Iganga, Uganda—a reflection of differences in policies regardingwithdrawal of chloroquine? *Acta Trop*. 121, 148–151.

Kaslow, D. C. (1994). Progress toward a transmission-blocking vaccine. In: Good MF, Saul J, editors. *Molecular immunological consideration in malaria vaccine development*. London: CRC Press, Boca Raton FL; p. 209-44.

Kolawole, O. M., Olugbenga, A. M., Yetunde, A. O., and Tolulope O. O. (2016). Population Genomics Diversity of *Plasmodium falciparum* in Malaria Patients Attending Okelele Health Centre, Okelele, Ilorin, Kwara State, Nigeria. *African Health Sciences* 16(3): 704–711.

Kolawole, O., Ozokonkwo, O., and Mokuolu, O. (2014). Prevalence of *Plasmodium falciparum* Malaria Among Patients Attending University of Ilorin Teaching Hospital, Ilorin, Nigeria. *ARI*. 11(3): 2063 – 2070.

Koleala, T., Karl, S., Laman, M., Moore, B. R., Benjamin, J., Barnadas, C., Robinson,L. J., Kattenberg, J. H., Javati, S., Wong, R.P. M., Rosanas-Urgell, A., Betuela, I., Siba, P. M., Mueller,I. andDavis T. M.,(2015). Temporal changes in Plasmodium falciparum anti-malarial drug sensitivity in vitro and resistance-associated genetic mutations in isolates from Papua New Guinea. *Malaria Journal*, *14*, 37. https://doi.org/10.1186/s12936-015-0560-3

Kosuwin, R., Putaporntip, C., Tachibana, H. and Jongwutiwes, S. (2014). Spatial variation in genetic diversity and natural selection on the thrombospondin-related adhesive protein locus of *Plasmodium vivax* (PvTRAP). *PloS One*, *9*(10).

Kublin, J.G., Cortese, J.F., Njunju, E.M., Mukadam, R.A.G., Wirima, J.J., Kazembe, P.N.,Djimdé, A.A., Kouriba, B., Taylor, T.E., and Plowe, C. V.(2003). Reemergence ofchloroquine-sensitive *Plasmodium falciparum* malaria after cessation ofchloroquine use in Malawi. *J. Infect. Dis*. 187, 1870–1875.

Kuile, F. O. T., Terlouw, D. J., Phillips-Howard, P. A., Hawley, W. A., Friedman, J. F., Kariuki, S. K., Shi, Y. P., Kolczak, S. M., Lal, A. A., Vulule, M. J. and Nahlen, L. B. (2003). Reduction of Malaria During Pregnancy by Permethrin-Treated Bed Nets in an Area of Intense Perennial Malaria Transmission in Western Kenya. *Am J Trop Med Hyg*. 68:50–60.

Kumar, D., Dhiman, S., Rabha, B., Goswami, D., Deka, M., Singh, L., Baruah, I. and Veer, V (2014). Genetic polymorphism and amino acid sequence variation in *Plasmodium falciparum* GLURP R2 repeat region in Assam, India, at an interval of five years. *Malaria Journal*, *13*(1), 450. https://doi.org/10.1186/1475-2875-13-450

Laufer, M.K., Thesing, P.C., Eddington, N.D., Masonga, R., Dzinjalamala, F.K., Takala,S.L., Taylor, T.E. and Plowe, C.V.(2006). Return of chloroquine antimalarial efficacyin Malawi. *N. Engl. J. Med.* 355, 1959–1966.

Lee, S. M., Wu, C.K., Plieskatt, J., McAdams, D. H., Miura, K., Ockenhouse, C. and King, C. R. (2016). Assessment of Pfs25 expressed from multiple soluble expression platforms for use as transmission-blocking vaccine candidates. *Malaria Journal*, *15*(1), 405. https://doi.org/10.1186/s12936-016-1464-6

Lim, S. S., Fullman, N., Stokes, A., Ravishankar, N., Masiye, F., Murray, C. J. L. and Gakidou, E. (2011). Net Benefits: A Multicountry Analysis of Observational Data Examining Associations between Insecticide-Treated Mosquito Nets and Health Outcomes. *PLoS* Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3167799/

Li, P., Zhao, Z., Xing, H., Li ,W., Zhu, X., Cao, Y., Yang, Z., Sattabongkot, J., Yan, G., Fan Q. and Cu, L. (2016). *Plasmodium malariae* and *Plasmodium ovale* infections in the China-Myanmar border area. *Malar. J.*; 15:557.

Lo, E., Nguyen, J., Oo, W., Hemming-Schroeder, E., Zhou, G., Yang, Z., Cui, L. and Yan, G. (2016). Examining Plasmodium falciparum and P. vivax clearance subsequent to antimalarial drug treatment in the Myanmar-China border area based on quantitative real-time polymerase chain reaction. *BMC Infectious Diseases*, *16*. https://doi.org/10.1186/s12879-016-1482-6

Lobo, C. A., Dhar, R.and Kumar, N. (1999). Immunization of mice with DNA-based Pfs25 elicits potent malaria transmission- blocking antibodies. *Infect Immun*; *67*: 1688-93.

Lorenz, V., Karanis, G., and Karanis, P. (2014). Malaria vaccine development and how external

forces shape it: An overview. *International Journal of Environmental Research and Public Health*, *11*(7), 6791–6807. https://doi.org/10.3390/ijerph110706791

Malaria Vaccine Initiative (2013). *www.malariavaccine.org*, Accessed on April 25th August 2016

Malaria Vaccine Technology Roadmap August (2006). http://www.malariavaccine.org. Accessedon 19th April 2016.

Malmberg, M., Ngasala, B., Ferreira, P.E., Larsson, E., Jovel, I., Hjalmarsson, A.,Petzold, M., Premji, Z., Gil, J.P., Bjorkman, A., and Martensson, A.(2013). Temporaltrends of molecular markers associated with artemether-lumefantrinetolerance/resistance in Bagamoyo district, Tanzania. *Malar. J*. 12, 103.

Manego, R. Z., Mombo-Ngoma, G., Witte, M., Held, J., Gmeiner, M., Gebru, T., Tazemda, B., Mischlinger, J., Groger, M., Lell, B.,. Adegnika, A. A., Agnandji, S. T., Kremsner, P. G., Mordmüller, B., Ramharter, M. and MatsieguiP. B. (2017). Demography, maternal health and the epidemiology of malaria and other major infectious diseases in the rural department Tsamba-Magotsi, Ngounie Province, in central African Gabon. *BMC Public Health*.;17:130.

Mang'era, C.M., Mbai, F.N., Omedo, I.A., Mireji, P.O., and Omar, S.A., (2012). Changes ingenotypes of *Plasmodium falciparum* human malaria parasite followingwithdrawal of chloroquine in Tiwi, Kenya. *Acta Trop*. 123, 202–207.

Marks, F., Evans, J., Meyer, C. G., Browne, E. N., Flessner, C., Von Kalckreuth, V.,Eggelte, T. A., Horstmann, R. D. and May, J. (2005). High prevalence of markers for sulfadoxine and pyrimethamine resistance in Plasmodium falciparum in the absence of drug pressure in the Ashanti Region of Ghana. *Antimicrobial Agents and Chemotherapy*, *49*(3), 1101–1105. https://doi.org/10.1128/AAC.49.3.1101-1105.2005

Matuschewski, K., Nunes, A. C., Nussenzweig, V. and Ménard, R. (2002). Plasmodium sporozoite invasion into insect and mammalian cells is directed by the same dual binding system. *EMBO Journal*, *21*(7), 1597–1606. https://doi.org/10.1093/emboj/21.7.1597

May, J., Mockenhaupt, F. P., Ademowo, O. G., Falusi, A. G., Olumese, P. E., Bienzle, U.*et al*.(1999). High rate of mixed and subpatent malarial infections in southwest Nigeria. *Am J Trop Med Hyg.*;61:339–43.

Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R. V. *et al.* (2015). A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. *Nature*, *520*(7549), 683–687. https://doi.org/10.1038/nature14412

Mekonnen, S.K., Aseffa, A., Berhe, N., Teklehaymanot, T., Clouse, R.M., Gebru, T.,Medhin, G., Velavan, T.P., (2014). Return of chloroquine-sensitive *Plasmodium falciparum* parasites and emergence of chloroquine-resistant *Plasmodium vivax*in Ethiopia. *Malar. J*. 13, 244.

Menard, D., Barnadas, C., Bouchier, C.,Henry-Halldin, C., Gray, L. R., Ratsimbasoa, A., Thonier, V., Carod, J., Domarle, O., Colin, Y., Bertrand, O., Picot, J., King C. L., Grimberg, B. T., Mercereau-Puijalon, O. and. Zimmerman P. A. (2010). *Plasmodium vivax* clinical malaria is commonly observed in Duffy-negative Malagasy people. *Proc Natl Acad Sci US* ;107:5967–71.

Mendes, C., Dias, F., Figueiredo, J.,Vicenta Gonzalez Mora, V. G., Cano, J., de Sousa, B.,do Rosário, V. E., Benito, A., Berzosa, P. and Arez A. P. (2011). Duffy negative antigen is no longer a barrier to *Plasmodium vivax*—molecular evidences from the African West Coast (Angola and Equatorial Guinea). *PLoS Negl Trop Dis*; 5:e1192.

Miller, L. H., Mason, S. J., Clyde, D. F., and McGinniss, M. H.(1976). The resistance factor to *Plasmodium vivax* in Blacks. The Duffy-blood-group genotype, FyFy. *N Engl J Med*; 295:3024.

Mohammed, A., Ndaro, A., Kalinga, A., Manjurano, A., Mosha, J.F., Mosha, D.F., vanZwetselaar, M., Koenderink, J.B., Mosha, F.W., Alifrangis, M., Reyburn, H., Roper,C. and Kavishe, R. A. (2013). Trends in chloroquine resistance marker, Pfcrt-K76Tmutation ten years after chloroquine withdrawal in Tanzania. *Malar. J.* 12, 415

Morgan, J. C., Irving, H., Okedi, L. M., Steven, A. and Wondji, C. S. (2010). Pyrethroid resistance in an Anopheles funestus population from Uganda. *PLoS One*, *5*(7), e11872

Morrison, D. (2010). A history of PCR. *Australian Systematic Botany Society Newsletter*, *142*(March), 3–6.

Munshi, A. (2012). Dna Sequencing – Methods And Applications. (T. Smiljanic, Ed.) (free online). Croatia: Bojan Rafaj. https://doi.org/10.5772/2158

Murray, C.J.L., Rosenfeld, L.C., Lim, S.S., Andrews, K.G., Foreman, K.J, Haring, D., Fullman, N., Naghavi, M., Lozano, R., and Lopez, A.D. (2010): Global malaria mortality between 1980 and 2010: a systematic analysis. *The Lancet*, **379** (9814): 413-431.

Mvi and Gsk. (2015). Fact sheet: The RTS,S Malaria Vaccine Candidate (Mosquirix TM), 4. Retrieved from http://www.malariavaccine.org/files/MVI-GSK-RTSSfactsheetFINAL-web.pdf

Mvumbi, D. M., Kayembe, J.-M., Situakibanza, H., Bobanga, T. L., Nsibu, C. N., Mvumbi, G. L.,Melin, P., Mol, P. D. and Hayette, M. (2015). Falciparum malaria molecular drug resistance in the Democratic Republic of Congo: a systematic review. *Malaria Journal*, *14*, 354. https://doi.org/10.1186/s12936-015-0892-z

Mwai, L., Ochong, E., Abdirahman, A., Kiara, S.M., Ward, S., Kokwaro, G., Sasi, P.,Marsh, K., Borrmann, S. and Mackinnon, M., (2009). Chloroquine resistance beforeand after its withdrawal in Kenya. *Malar. J.* 8, 106

Nneji, C. M., Adedapo, A. D. A. and Okorie, P. N. (2015). iMedPub Journals Chloroquine Resistance and Host Genetic Factors among Nigerian Children with Uncomplicated P . falciparum Infection Abstract, *7*(4), 1–9.

Noland, G. S., Graves, P. M., Sallau, A., Eigege, A., Emukah, E., Patterson, A. E., Ajiji, J., Okorofor, I., Oji, O. U., Umar, M., Alphonsus, K., Damen, J.,Ngondi, J., Ozaki, M., Cromwell, E., Obiezu, J., Eneiramo, S., Okoro, C., McClintic-Doyle, R., Oresanya, O., Miri, E., Emerson P. M. and RichardsJr F. O. (2014). Malaria prevalence, anemia and baseline intervention coverage prior to mass net distributions in Abia and Plateau States, Nigeria. *BMC Infect. Dis.*;14:168.

Nosten, F., McGready, R., Simpson, J. A., Thwai, K. L., Balkan, S., Cho, T., Hkirijaroen, L., Looareesuwan, S and White, N. J. (1999). Effects of *Plasmodium vivax* malaria in pregnancy. *Lancet* 354, 546–549

Oeuvray, C., Theisen, M., Rogier, C., Trape, J. F., Jepsen, S., and Druilhe, P. (2000). Cytophilic immunoglobulin responses to *Plasmodium falciparum* glutamate- rich protein are correlated with protection against clinical malaria in Dielmo, Senegal. *Infection and Immunity*, *68*(5), 2617–2620. https://doi.org/10.1128/IAI.68.5.2617-2620.2000

Ohashi, J., Suzuki, Y., Naka, I., Hananantachai, H., and Patarapotikul, J. (2014). Diversifying selection on the thrombospondin-related adhesive protein (TRAP) gene of *Plasmodium falciparum* in Thailand. *PLoS ONE*, *9*(2), e90522.

Ohrt, C., Purnomo, Sutamihardja, M. A., Tang, D. and Kain, K. C. (2002): Impact of microscopy error on estimates of protective efficacy in malaria-prevention trials.*J Infect Dis*, 186:540–546.

Ojurongbe, O., Ogungbamigbe, T. O., Fagbenro-Beyioku, A. F., Fendel, R., Kremsner, P. G. and Kun, J. F. (2007). Rapid detection of Pfcrt and Pfmdr1 mutations in *Plasmodium falciparum* isolates by FRET and in vivo response to chloroquine among children from Osogbo, Nigeria. *Malaria Journal*, 6, 41.https://doi.org/10.1186/1475-2875-6-41

Olasehinde, G. I., Yah, C. S., Singh, R.,Ojuronbge, O. O., Ajayi, A. A., Valecha, N., Abolaji, A. O. and AdeyebaA. O. (2012). Genetic Diversity of Plasmodium falciparum field Isolates from South Western Nigeria. *African Health Sciences* 12 (3): 355-361.

Olawumi, H.O., Fadeyi, A., Babatunde, S.k., Akanbi, A. A., Babatunde, A. S., Sani, M. A and Aderibigbe, S. A. (2015). Malaria Parasitaemia among Blood Donors in Ilorin, Nigeria. *African Journal of Infectious Diseases, 9*(1), 10-13

Olukosi, Y. A., Oyebola, M. K., Ajibaye, O., Aina, O. O., Agomo, C. O., Iwalokun, B. A., Akindele, S. K., Enya, N. V. and Okoh, H. I.(2014). Persistence of markers of chloroquine resistance among *P. falciparum* isolates recovered from two Nigerian communities. *MWJ* 5:3 Pp 3-6

Olusegun, M. A. (2015). Effect of Age on Prevalence of Malaria and Oxidative Stress in Children. *British Microbiology Research Journal*,8 (1): 379-385.

Onwuasigwe C. (2004). Determination of sample size. In: Medical Research Project: A practical guide. Enugu: Eldemak Publishers,111-118

Onwujekwe, O., Akpala, C., Ghasi, S., Shu E. and Okonkwo, P. (2000). How do rural households perceive and prioritise malaria and mosquito nets? A study in five communities of Nigeria. Public Health [Internet].;114. Available from: http://dx.doi.org/10.1016/S0033-3506(00)003735

Onwujekwe, O., Etiaba, E., Uguru, N., Uzochukwu, B. and Adjagba, A. (2014). Towards making efficient use of household resources for appropriate prevention of malaria: investigating households' ownership, use and expenditures on ITNs and other preventive tools in Southeast Nigeria. *BMC Public Health*; 14:315

Otsyula, N., Angov, E., Bergmann-Leitner, E., Koech, M., Khan, F., Bennett, J. *et al.* (2013). Results from tandem Phase 1 studies evaluating the safety, reactogenicity and immunogenicity of the vaccine candidate antigen Plasmodium falciparum FVO merozoite surface protein-1 (MSP1(42)) administered intramuscularly with adjuvant system AS01. *Malaria Journal*, *12*(1), 29. https://doi.org/10.1186/1475-2875-12-29

Oyebola,M. K., Idowu, E. T., Olukosi, Y. A., Iwalokun, B. A., Agomo, C. O., Ajiboye, O. O. ,Tola M, and Otubanjo, A. O. (2014). Genetic diversity and complexity of *Plasmodium falciparum* infections inLagos, Nigeria. *Asian Pac J Trop Biomed*; 4(Suppl 1): S87-S91

Oyedeji, S. I., Awobode, H. O. and Kun J.J. (2013a). Limited Genetic Diversity and low Multiplicity of *Plasmodium falciparum* infections in Children with Severe Malaria inLafia, North-Central Nigeria. *J Exp Clin Med*; 5(4): 143 -147

Oyedeji, S. I., Henrietta O. A., Chiaka, A. and Jurgen K. (2013b). Genetic Diversity of *Plasmodium falciparum* Isolates from Naturally Infected Children in North – Central Nigeria Using the Merozoite Surface Protein-2 as Molecular Marker. *Asian Pacific Journal of Tropical Medicine* 6(8): 589-594

Parida, P., Sarma, K., Borkakoty, B. and Mohapatra, P. K. (2016). Structure and Functional Differentiation of PfCRT Mutation in Chloroquine Resistance (CQR) in Plasmodium falciparum Malaria. In *Current Topics in Malaria*. InTech. https://doi.org/10.5772/64659

Petersen, T. N., Brunak, S., Heijne, G. and Nielsen, H. (2011). SignalP4.0: discriminating signal peptides from transmembrane regions. *Nat Methods*, *8*. https://doi.org/10.1038/nmeth.1701

Phyo, A.P., Nkhoma, S., Stepniewska, K., Ashley, E.A., Nair, S., McGready, R., ler Moo,C., Al-Saai, S., Dondorp, A.M. and Lwin, K. M.(2012). Emergence ofartemisinin-resistant malaria on the western border of Thailand: alongitudinal study. *Lancet* 379, 1960–1966.

Pratt-Riccio, L. R., Lima, J. C., Carvalho, L. J. M., Theisen, M., Espíndola-Mendes, E. C., Santos, F., Oliveira-Ferreira, J., Goldberg, A. C., Daniel-Ribeiro, C. T. and Banic, D. M. (2005). Antibody response profiles induced by *Plasmodium falciparum* glutamate-rich protein in naturally exposed individuals from a Brazilian area endemic for malaria. *American Journal of Tropical Medicine and Hygiene*, *73*(6), 1096–1103.

Pratt-Riccio, L. R., Perce-da-Silva, D. de S., Lima-Junior, J. da C., Theisen, M., Santos, F., Daniel-Ribeiro, C. T., Oliveira-Ferreira, J.and Banic, D. M. (2013). Genetic polymorphisms in the glutamate-rich protein of *Plasmodium falciparum* field isolates from a malaria-endemic area of Brazil. *Memorias Do Instituto Oswaldo Cruz*, *108*(4), 523–528. https://doi.org/10.1590/0074-0276108042013022

Preechapornkul, P., Imwong, M., Chotivanich, K., Pongtavornpinyo, W., Dondorp, A. M., Day, N. P. J., White, N. J. and Pukrittayakamee, S. (2009). *Plasmodium falciparum* Pfmdr1 amplification,mefloquine resistance, and parasite fitness. *Antimicrobial Agents and Chemotherapy*, *53*(4), 1509–1515. https://doi.org/10.1128/AAC.00241-08

Price, R. N., Cassar, C., Brockman, A., Duraisingh, M., van Vugt, M., White, N. J., Nosten, F. and Krishna,S. (1999). The pfmdr1 gene is associated with a multidrug-resistant phenotype in Plasmodium falciparum from the western border of Thailand. *Antimicrobial Agents and Chemotherapy*, *43*(12), 2943–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10582887

Price, R. N., Uhlemann, A. C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S., White, N. J., Nosten, F. and Krishna, S. (2004). Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. *The Lancet*, *364*(9432), 438–447. https://doi.org/10.1016/S0140-6736(04)16767-6

Pullan, R. L., Bukirwa, H., Snow, R. W. and Brooker, S. (2010). Heritability of Plasmodium Parasite Density in a Rural Ugandan Community. *Am J Trop Med Hyg*.;83:990–5.

Rao, A. and Tapale, S. (2013). A study on dihydrofolate reductase and its inhibitors: a review. *International Journal of Pharmaceutical* *4*(7), 2535–2547. https://doi.org/10.13040/IJPSR.0975-8232.4(7).2535-47

Rattanachuen, W., Jönsson, M., Swedberg, G. and Sirawaraporn, W. (2009). Probing the roles of non-homologous insertions in the N-terminal domain of Plasmodium falciparum hydroxymethylpterin pyrophosphokinase–dihydropteroate synthase. *Molecular and Biochemical Parasitology*, *168*(2), 135–142. https://doi.org/10.1016/j.molbiopara.2009.07.005

Reiter, P. (2008). Global warming and malaria: knowing the horse before hitching the cart. *Malaria Journal*, *7 Suppl 1*(Suppl 1), S3. https://doi.org/10.1186/1475-2875-7-S1-S3

Remans, T., Keunen, E., Bex, G. J., Smeets, K., Vangronsveld, J. and Cuypers, A. (2014). Reliable gene expression analysis by reverse transcription-quantitative PCR: reporting and minimizing the uncertainty in data accuracy. *The Plant Cell*, *26*(10), 3829–37. https://doi.org/10.1105/tpc.114.130641

Rhee, M., Sissoko, M., Perry, S., McFarland, W., Parsonnet, J. and Doumbo, O. (2005). Use of insecticide-treated nets (ITNs) following a malaria education intervention in Piron, Mali: a control trial with systematic allocation of households. *Malaria Journal*; 4:35.

Richie, T. L., Billingsley, P. F., Sim, B. K. L., James, E. R., Chakravarty, S., Epstein, J. E.Lyke, K. E., Mordmüller, B., Alonso, P., Duffy, P. E., Doumbo, O. K., Sauerwein, R. W., Tanner, M., Abdulla, S., Kremsner, P. G., Seder, R. A. and Hoffman, S. L. (2015). Progress with *Plasmodium falciparum* sporozoite (PfSPZ)-based malaria vaccines. *Vaccine*, *33*(52), 7452–7461. https://doi.org/10.1016/j.vaccine.2015.09.096

Ridley, R. G. (2003). Malaria: To kill a parasite. *Nature*, *424*(6951), 887–889. https://doi.org/10.1038/424887a

Riley, E. M., and Stewart, V. A. (2013). Immune mechanisms in malaria: new insights in vaccine development. *Nature Medicine*, *19*(2), 168–178. https://doi.org/10.1038/nm.3083

Rogers, W. O., Malik, A., Mellouk, S., Nakamura, K., Rogers, M. D., Szarfman, A. Gordon, D. M.,Nussler, A. K., Aikawa, M. and Hoffman, S. L. (1992). Characterization of *Plasmodium falciparum* sporozoite surface protein 2. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(19), 9176–80. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1409621

Rougemont, M., Van Saanen, M., Sahli, R., Hinrikson, H. P., Bille, J. and Jaton, K. (2004). Detection of four Plasmodium species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. *J Clin Microbiol.*;42:5636–43.

Ryan, J. R., Stoute, J. A., Amon, J., Dunton, R. F., Mtalib, R., Koros, J., Owour, B., Luckhart, S., Wirtz, R. A., Barnwell, J. W. and Rosenberg, R. (2006). Evidence for transmission of *Plasmodium vivax* among a Duffy antigen negative population in Western Kenya. *Am J Trop Med Hyg*; 75:575–81.

Sá, J. M., Twu, O., Hayton, K., Reyes, S., Fay, M. P., Ringwald, P. and Wellems, T. E. (2009). Geographic patterns of Plasmodium falciparum drug resistance distinguished by differential responses to amodiaquine and chloroquine. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(45), 18883–9. https://doi.org/10.1073/pnas.0911317106

Saha, P., Swagata, G. and Ardhendu, K. M.(2016). Genetic Diversity and Multiplicity of Infection of *Plasmodium falciparum* Isolates from Kolkota, West Bengal, India. Infection, Genetics and Evolution: *Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious* Diseases 43: 239- 244.

Sardá, V., Kaslow, D. C. and Williamson, K. C. (2009). Approaches to malaria vaccine development using the retrospectroscope. *Infection and Immunity*. https://doi.org/10.1128/IAI.00122-09

Sharma, J., Dutta, P., Khan, S. A., Soni, M. and Mahanta, J. (2014). Detection of point mutation in plasmodium falciparum atpase6 gene associated with artemisinin resistance from assam and arunachal pradesh. *Journal of Vector Borne Diseases*, *51*(4), 282–285.

Sharma, S., and Pathak, S. (2008). Malaria vaccine: a current perspective. *Journal of Vector Borne Diseases*, *45*(1), 1–20.

Shimp, R. L., Rowe, C., Reiter, K., Chen, B., Nguyen, V., Aebig, J.,Rausch, K. M., Kumar, K., Wu, Y., Jin, A. J., Jones, D. S. and Narum, D. L.(2013). Development of a Pfs25-EPA malaria transmission blocking vaccine as a chemically conjugated nanoparticle. *Vaccine*, *31*(28), 2954-2962.

Sidhu, A., Verdier-Pinard, D. and Fidock, D. A. (2002). Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. *Science*, *298*(5591), 210–213. https://doi.org/10.1126/science.1074045.Chloroquine

Sing, M., Brown, G. and Rogerson, S. J. (2013). Ownership and use of insecticide-treated nets during pregnancy in sub-Saharan Africa: a review. *Malaria Journal* 12:268.

Snounou, G. (2002). Genotyping of Plasmodium species using Nested PCR. *Methods in Molecular Medicine, 72:103- 16.*

Snounou, G. and Singh, B. (2002). Nested PCR analysis of Plasmodium parasites. Methods Mol Med. 72:189–203

Snounou, G., Zhu, X., Siripoon, N., Jarra, W., Thaithong, S., Brown, K.N. and Viriyakosol, S.(1999). Biased distribution of MSP-1 and MSP-2 allelic variants in *Plasmodium falciparum* populations in Thailand. *Trans. R. Soc. Trop. Med. Hyg*. 93, 369–374

Song, G., Koksal, A. C., Lu, C. and Springer, T. A. (2012). Shape change in the receptor for gliding motility in Plasmodium sporozoites. *Proceedings of the National Academy of Sciences*, *109*(52), 21420–21425. https://doi.org/10.1073/pnas.1218581109

Steenkeste, N., Rogers, W. O., Okell, L., Jeanne, I., Incardona, S., Duval, L., Chy, S., Hewitt, S., Chou, M., Socheat, D., Babin, F., Ariey F. and Rogier,C. (2010) Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Rattanakiri province, Cambodia: implication for malaria elimination. *Malaria Journal*, 9:108

Stoute, J. A., Slaoui, M., Heppner, D. G., Momin., P., Kester, K. E, Des- mons, P., Wellde, B. T., Garçon, N., Krzych, U., Marchand, M., Bal- lou, W. R. and Cohen, J. D. (1997). A preliminary evaluation of a re- combinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. *N Engl J Med* 336: 86–91.

Stowers, A. W., Keister, D. B., Muratova, O. and Kaslow, D. C. (2000). A Region of Plasmodium falciparumAntigen Pfs25 That Is the Target of Highly Potent Transmission-Blocking Antibodies. *Infection and immunity*, *68*(10), 5530-5538

Tamirat, G., Asrat, H., Martin, P. G. and Jürgen F. J. Kun. (2005). Molecular Surveillance of Mutations in Dihydrofolatereductase and Dihyropteroate Synthase Genes of *Plasmodium falciparum* in Ethiopia. *Am. J. Trop. Med. Hyg*., 73(6), pp. 1131–1134

The RTS, S. C. T. P. (2014). Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. *PLoS Medicine*, *11*(7), e1001685. https://doi.org/10.1371/journal.pmed.1001685

Theisen, M., Soe, S., Oeuvray, C., Thomas, A. W., Vuust, J., Danielsen, S.Jepsen, S. and Druilhe, P. (1998). The glutamate-rich protein (GLURP) of *Plasmodium falciparum* is a target for antibody-dependent monocyte-mediated inhibition of parasite growth in vitro. *Infection and Immunity*, *66*(1), 11–17. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9423833

Thomsen, T.T., Madsen, L.B., Hansson, H.H., Tomas, E.V., Charlwood, D., Bygbjerg,I. C. and Alifrangis, M., (2013). Rapid selection of *Plasmodium falciparum* chloroquineresistance transporter gene and multidrug resistance gene-1 haplotypesassociated with past chloroquine and present artemether-lumefantrine use inInhambane District, southern Mozambique. *Am. J. Trop. Med. Hyg.* 88, 536–541.

Tishkoff, S. A., Varkonyi, R., Cahinhinan, N., Abbes, S., Argyropoulos, G., Destro-Bisol, G., Drousiotou, A., Dangerfield, B., Lefranc, G., Loiselet, J., Piro, A., Stoneking, M., Tagarelli, A., Tagarelli, G., Touma, E. H., Williams, S. M. and Clark, A. G. (2001): Haplotype diversity and linkage disequilibrium at human G6PD: recent origin of alleles that confer malarial resistance. *Science*, 293: 455–462.

Turner, L., Wang, C. W., Lavstsen, T., Mwakalinga, S. B., Sauerwein, R. W., Hermsen, C. C. and Theander, T. G. (2011). Antibodies against PfEMP1, RIFIN, MSP3 and GLURP are acquired during controlled *Plasmodium falciparum* malaria infections in naïve volunteers. *PLoS ONE*, *6*(12), e29025. https://doi.org/10.1371/journal.pone.0029025

Umaru, M. L. and Uyaiabasi, G. N. (2015). Prevalence of Malaria in Patients Attending the General Hospital Makarfi, Makarfi Kaduna – State, North-Western Nigeria. *American Journal of Infectious Diseases and Microbiology*, 3(1), 1-5.

Valones, M. A. A., Guimarães, R. L., Brandão, L. A. C., De Souza, P. R. E., De Albuquerque Tavares Carvalho, A. and Crovela, S. (2009). Principles and applications of polymerase chain reaction in medical diagnostic fields: A review. *Brazilian Journal of Microbiology*, *40*(1), 1–11. https://doi.org/10.1590/S1517-83822009000100001

Valupadasu, M. and Mateti, U. (2012). Advanced malarial vaccines: A promising approach in the treatment of malaria. *Systematic Reviews in Pharmacy*, *3*(1), 31–36. https://doi.org/http://dx.doi.org/10.4103/0975-8453.107136

Viana, G. M. R., Machado, R. L. D., Calvosa, V. S. P. and Póvoa, M. M. (2006). Mutations in the pfmdr1, cg2, and pfcrt genes in Plasmodium falciparum samples from endemic malaria areas in Rondonia and Pará State, Brazilian Amazon Region. *Cadernos de Saude Publica / Ministerio Da Saude, Fundacao Oswaldo Cruz, Escola Nacional de Saude Publica*, *22*(12), 2703–2711. https://doi.org/10.1590/S0102-311X2006001200019

Vinayak, S., Mittra, P and Sharma, Y. D. (2006). Wide variation in microsatellite sequences within each Pfcrt mutant haplotype. *Mol Biochem Parasitol* 147: 101–108.

Waller, K. L., Muhle, R. A., Ursos, L. M., Horrocks, P., Verdier-Pinard, D., Sidhu, A. B. S.,Fujioka, H., Roepe, P. D. and Fidock, D. A. (2003). Chloroquine resistance

modulated in vitro by expression levels of the Plasmodium falciparum chloroquine resistance transporterJournal of Biological Chemistry, 278(35), 33593-33601.

White, N.J. (2003). Malaria. In Manson's Tropical Diseases (Cook, G.C. and Zumla, A., eds), pp. 1205–1295.

WHO (2011). The technical basis for coordinated action against insecticide resistance: preserving the effectiveness of modern malaria vector control. Report of a meeting 4-6 May 2010. 46pp. Geneva: Global Malaria Programme, WHO.

WHO (2012). WHO Evidence Review Group : Intermittent Preventive Treatment of malaria in pregnancy ( IPTp ) with Sulfadoxine - Pyrimethamine ( SP ), (September), 1–17.

WHO (2013). fact sheet no. 94. *Geneva: World Health Organization*. http://www.who.int/mediacentre/factsheets/fs094/en/. Accessed on 3rd April 2016.

WHO (2016). Fact Sheet: World Malaria Report. Retrieved February 15, 2017, from http://www.who.int/malaria/media/world-malaria-report-2016/en/

WHO | Insecticide-treated nets to reduce the risk of malaria in pregnant women [Internet]. WHO. [cited 2017 Jan 19]. Available from: http://www.who.int/elena/titles/bednets_malaria_pregnancy/en/

WHO, (2011). World malaria report. Geneva: World Health Organization.

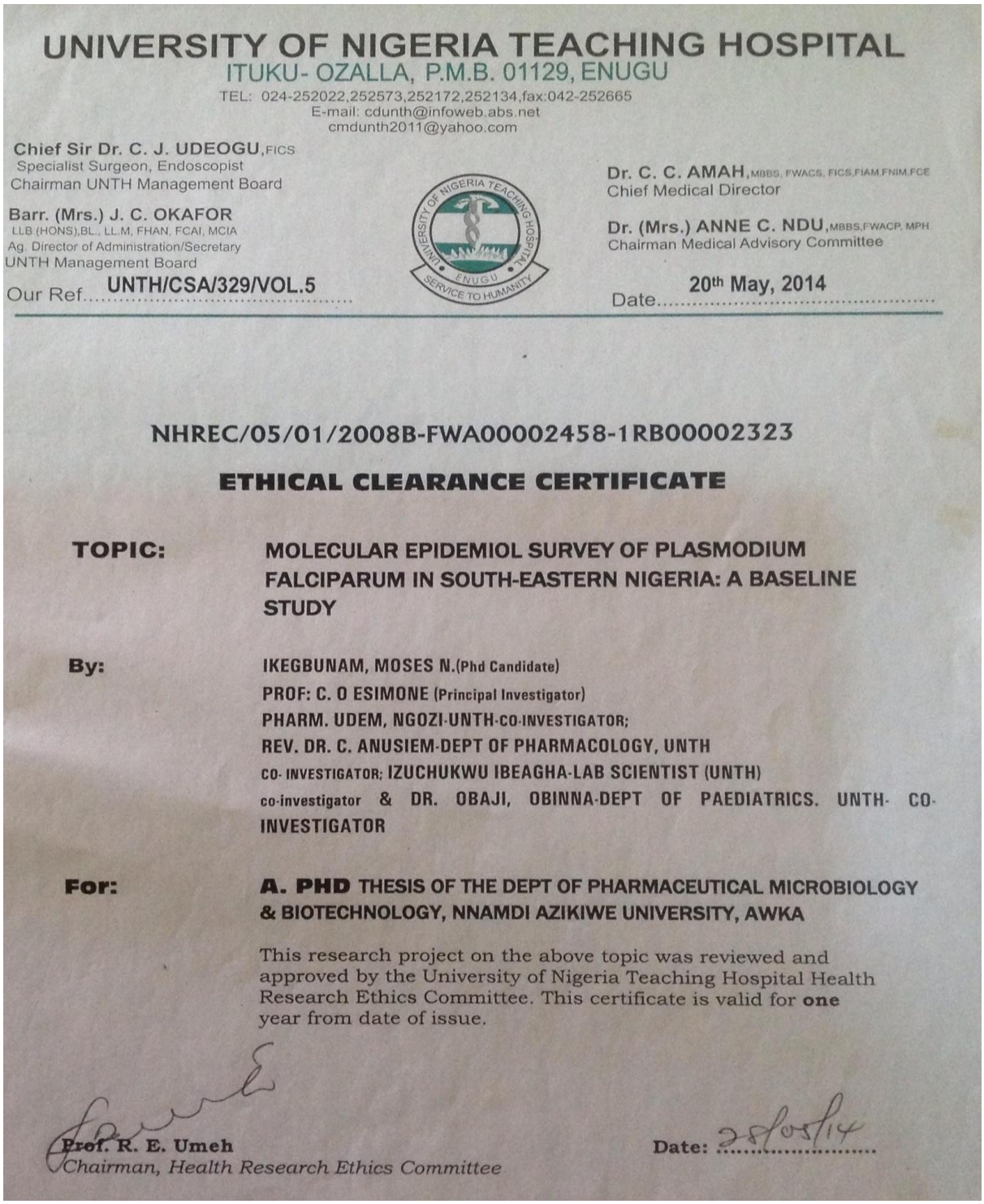
Wurtz, N., Mint, L. K., Bogreau, H.,Pradines, B., Rogier, C., Boukhary, A. O. M. S., Hafid, J. E., Salem, M. S. O. A., Trape, J. F., Basco, L. K. and Briolant, S.,(2011). Vivax malaria in Mauritania includes infection of a Duffy-negative individual. *Malaria journal*, *10*(1), p.336

Zakeri, S., Bereczky, S., Naimi, P., Gil, J.P., Djadid, N.D., Fa¨rnert, A., Snounou, G. and Bjo¨rkman, A.(2005). Multiple genotypes of the merozoite surface proteins 1 and 2 in Plasmodium falciparum infections in a hypoendemic area in Iran. Tropical Med. Int. Health 10 (10), 1060–1064. http://dx.doi.org/10.1111/j.1365-3156.2005.01477.x.

Zakeri, S., Hemati, S., Pirahmadi, S., Afsharpad, M., Raeisi, A. and Djadid, N. D. (2012). Molecular assessment of atpase6 mutations associated with artemisinin resistance among unexposed and exposed Plasmodium falciparum clinical isolates to artemisinin-based combination therapy. Malaria Journal, 11, 373. https://doi.org/10.1186/1475-2875-11-373

Zeeshan, M., Alam, M. T., Vinayak, S., Bora, H., Tyagi R. K., Alam, M. S., Choudhary, V., Mittra, P., Lumb, V., Bharti, P. K. and Udhayakumar, V. (2012) Genetic Variation in the Plasmodium falciparum Circumsporozoite Protein in India and Its Relevance to RTS,S Malaria Vaccine. *PLoS ONE 7(*8): e43430. doi:10.1371/journal.pone.0043430

# Appendix



UNIVERSITY OF NIGERIA TEACHING HOSPITAL
ITUKU- OZALLA, P.M.B. 01129, ENUGU
TEL: 024-252022,252573,252172,252134,fax:042-252665
E-mail: cdunth@infoweb.abs.net
cmdunth2011@yahoo.com

Chief Sir Dr. C. J. UDEOGU, FICS
Specialist Surgeon, Endoscopist
Chairman UNTH Management Board

Barr. (Mrs.) J. C. OKAFOR
LLB (HONS),BL., LL.M, FHAN, FCAI, MCIA
Ag. Director of Administration/Secretary
UNTH Management Board

Dr. C. C. AMAH, MBBS, FWACS, FICS,FIAM,FNIM,FCE
Chief Medical Director

Dr. (Mrs.) ANNE C. NDU, MBBS,FWACP, MPH
Chairman Medical Advisory Committee

Our Ref. UNTH/CSA/329/VOL.5

Date. 20th May, 2014

NHREC/05/01/2008B-FWA00002458-1RB00002323

**ETHICAL CLEARANCE CERTIFICATE**

**TOPIC:** MOLECULAR EPIDEMIOL SURVEY OF PLASMODIUM FALCIPARUM IN SOUTH-EASTERN NIGERIA: A BASELINE STUDY

**By:** IKEGBUNAM, MOSES N.(Phd Candidate)
PROF: C. O ESIMONE (Principal Investigator)
PHARM. UDEM, NGOZI-UNTH-CO-INVESTIGATOR;
REV. DR. C. ANUSIEM-DEPT OF PHARMACOLOGY, UNTH
CO- INVESTIGATOR; IZUCHUKWU IBEAGHA-LAB SCIENTIST (UNTH)
co-investigator & DR. OBAJI, OBINNA-DEPT OF PAEDIATRICS. UNTH- CO-INVESTIGATOR

**For:** **A. PHD** THESIS OF THE DEPT OF PHARMACEUTICAL MICROBIOLOGY & BIOTECHNOLOGY, NNAMDI AZIKIWE UNIVERSITY, AWKA

This research project on the above topic was reviewed and approved by the University of Nigeria Teaching Hospital Health Research Ethics Committee. This certificate is valid for **one** year from date of issue.

**Prof. R. E. Umeh**
*Chairman, Health Research Ethics Committee*

**Date:** 28/05/14

**INFORMED CONSENT**

**Dear Sir/Madam**

I am IKEGBUNAM, MOSES NKECHUKWU, a PhD student of the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Agulu. I am carrying out a research project on

"**MOLECULAR EPIDEMIOLOGICAL SURVEY OF *Plasmodium falciparum* IN SOUTHEASTERN NIGERIA: A BASELINE STUDY."**

**Why the Study?**

In Nigeria, information about the prevalent strain of *Plasmodium falciparum* and the attendant parasite density is completely unavailable. This is a major impediment in the design and trial of not only malaria vaccines in Nigeria, but also in the holistic recommendation of suitable antimalarial to be used in our National Health Care Scheme. We hope that this study would bridge this gap particularly in South-eastern Nigeria.

**Procedure:**

Part of the blood submitted for malaria parasite examination would be spotted on a filter paper for onward DNA extraction.

**Participation and Participation fees**:

Participation is 100 percent free. You will not be paid nor expected to pay for participating.

**Confidentiality:**

Be assured that the outcome of the research would be treated with every confidentiality. Your identity will not be required for the study. Data analysis and presentation from this will be aggregate, and will not in any way reveal your identity.

**Risk/Benefit:**

There is no physical risk associated with this study as the same blood you submitted for diagnosis of malaria parasite would be used. The benefit is that you have positively contributed in the research that revealed the genetic diversity of *Plasmodium falciparum.*

**Your Consent**:

Now that the purpose and the procedure of the research have been explained to you, are you willing to participate in this study? YES (  ), NO (  )

Name and Signature of respondent  Name and Signature of Researcher

Date----------  Date----------

---

**QUESTIONNAIRE**

Dear All,

Please provide me with the information below. I am PhD a students working on "**MOLECULAR EPIDEMIOLOGICAL SURVEY OF *Plasmodium falciparum* IN SOUTHEASTERN NIGERIA: A BASELINE STUDY.**". Be assured that the information, if provided would be treated with every confidentiality. Please help me out.

**Instruction**: Please Tick "√ " in the box or write in the space provided against the option(s) appropriately.

<

**SOCIO-DEMOGRAPHIC PROFILE**

1. Sex: ☐ Male ☐ Female
2. Age: ☐ 5-14 ☐ 15-24 ☐ 25-34 ☐ 35-44 ☐ 45-54 ☐
3. Education level: Primary ☐ Secondary ☐ Tertiary ☐
4. Employment status: Employed ☐ Not employed ☐ Retired ☐ Students ☐
5. Religion: Christianity Muslim ☐ Others (specify) …….
6. Ethnicity: Igbo ☐ Yoruba ☐ Hausa ☐ Others (specify)…….
7. Residence: Rural/Village ☐ Urban/City ☐
8. Ownership of ITN: Yes ☐ No ☐
9. Use of net: Daily ☐ Occasionally ☐ Not at all ☐
10. Use of Antimalarials: a) When last did you take antimalarials?
    Less than 14days ☐ More than 14days ☐ Over a month ☐

    b) Which antimalarial did you take?
    SP ☐ Art ☐ ACT ☐ others (specify) _____

**Table 1: Descriptive Analysis of Parasiste density of various age ranges of Children Cohort**

**(0 -1) yr** **(2-5) yrs** **(6 - 10) yrs**

| | | | | | |
|---|---|---|---|---|---|
| Mean | 1811.22449 | Mean | 2191.683168 | Mean | 2417.959184 |
| Standard Error | 86.59977874 | Standard Error | 105.5157132 | Standard Error | 193.9249495 |
| Median | 1600 | Median | 2000 | Median | 1760 |
| Mode | 1200 | Mode | 2000 | Mode | 1360 |
| Standard Deviation | 857.2940711 | Standard Deviation | 1060.419794 | Standard Deviation | 1357.474646 |
| Sample Variance | 734953.1243 | Sample Variance | 1124490.139 | Sample Variance | 1842737.415 |
| Kurtosis | 1.288874513 | Kurtosis | 0.2263375 | Kurtosis | 0.658978576 |
| Skewness | 1.312534062 | Skewness | 0.68018619 | Skewness | 1.142206045 |
| Range | 3680 | Range | 5440 | Range | 5520 |
| Minimum | 720 | Minimum | 80 | Minimum | 720 |
| Maximum | 4400 | Maximum | 5520 | Maximum | 6240 |
| Sum | 177500 | Sum | 221360 | Sum | 118480 |
| Count | 98 | Count | 101 | Count | 49 |

**Table 2: Descriptive Analysis of Parasiste density with respect to use of mosquito nets in Children Cohort**

| *Always* | | *Sometimes* | | *Never* | |
|---|---|---|---|---|---|
| Mean | 2089.767 | Mean | 2178.462 | Mean | 2230.693 |
| Standard Error | 123.6737 | Standard Error | 334.0694 | Standard Error | 107.7958 |
| Median | 1680 | Median | 1760 | Median | 2000 |
| Mode | 1600 | Mode | 1440 | Mode | 3360 |
| Standard Deviation | 1146.903 | Standard Deviation | 1204.504 | Standard Deviation | 1083.334 |
| Sample Variance | 1315386 | Sample Variance | 1450831 | Sample Variance | 1173613 |
| Kurtosis | 1.251051 | Kurtosis | 0.797779 | Kurtosis | 1.148809 |
| Skewness | 1.299256 | Skewness | 1.300288 | Skewness | 0.900096 |
| Range | 5040 | Range | 3840 | Range | 5520 |
| Minimum | 720 | Minimum | 1040 | Minimum | 720 |
| Maximum | 5760 | Maximum | 4880 | Maximum | 6240 |
| Sum | 179720 | Sum | 28320 | Sum | 225300 |
| Count | 86 | Count | 13 | Count | 101 |

**Table 3: Descriptive Analysis of Parasiste density with respect to use of mosquito Repellents in Children Cohort**

| *Always* | | *Sometimes* | | *Never* | |
|---|---|---|---|---|---|
| Mean | 2367.826 | Mean | 2220.396 | Mean | 2071.111 |
| Standard Error | 209.2272 | Standard Error | 107.3785 | Standard Error | 153.7767 |
| Median | 2120 | Median | 2000 | Median | 1880 |
| Mode | 960 | Mode | 3360 | Mode | 1200 |
| Standard Deviation | 1419.048 | Standard Deviation | 1079.14 | Standard Deviation | 922.6601 |
| Sample Variance | 2013697 | Sample Variance | 1164544 | Sample Variance | 851301.6 |
| Kurtosis | 0.538699 | Kurtosis | 0.016132 | Kurtosis | -0.06484 |
| Skewness | 1.049332 | Skewness | 0.726454 | Skewness | 0.836153 |
| Range | 5520 | Range | 4960 | Range | 3360 |
| Minimum | 720 | Minimum | 720 | Minimum | 880 |
| Maximum | 6240 | Maximum | 5680 | Maximum | 4240 |
| Sum | 108920 | Sum | 224260 | Sum | 74560 |
| Count | 46 | Count | 101 | Count | 36 |

**Table 4: Ordinary ONE-WAY ANOVA of Age and Parasite Density of Children Cohort**

| | | | | | | |
|---|---|---|---|---|---|---|
| Table Analyzed | Age_Parasite Density | | | | | |
| ANOVA summary | | | | | | |
| F | 6.102 | | | | | |
| P value | 0.0026 | | | | | |
| P value summary | ** | | | | | |
| Are differences among means statistically significant? (P < 0.05) | Yes | | | | | |
| R square | 0.0469 | | | | | |
| Brown-Forsythe test | | | | | | |
| F (DFn | DFd) | 4.959 | (2 | 248) | | |
| P value | 0.0077 | | | | | |
| P value summary | ** | | | | | |
| Significantly different standard deviations? (P < 0.05) | Yes | | | | | |
| Bartlett's test | | | | | | |
| Bartlett's statistic (corrected) | 14.48 | | | | | |
| P value | 0.0007 | | | | | |
| P value summary | *** | | | | | |
| Significantly different standard deviations? (P < 0.05) | Yes | | | | | |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 1.35E+07 | 2 | 6.75E+06 | F (2 | 248) = 6.102 | P = 0.0026 |
| Residual (within columns) | 2.74E+08 | 248 | 1.11E+06 | | | |

| | | |
|---|---|---|
| Total | 2.88E+08 | 250 |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 3 |
| Number of values (total) | 251 |

| | |
|---|---|
| Table Analyzed | Mosquito net use_Parasite density |

ANOVA summary

| | |
|---|---|
| F | 4.977 |
| P value | 0.0002 |
| P value summary | *** |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.08056 |

Brown-Forsythe test

| | | | | |
|---|---|---|---|---|
| F (DFn | DFd) | 5.060 (5 | 284) | |
| P value | 0.0002 | | | |
| P value summary | *** | | | |
| Significantly different standard deviations? (P < 0.05) | Yes | | | |

Bartlett's test

| | |
|---|---|
| Bartlett's statistic (corrected) | 2253 |
| P value | < 0.0001 |
| P value summary | **** |
| Significantly different standard deviations? (P < 0.05) | Yes |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 4.20E+11 | 5 | 8.40E+10 | F (5 | 284) = 4.977 | P = 0.0002 |
| Residual (within columns) | 4.79E+12 | 284 | 1.69E+10 | | | |
| Total | 5.21E+12 | 289 | | | | |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 6 |
| | 223 |

| | |
|---|---|
| Number of values (total) | 290 |

**Table 5: Ordinary ONE-WAY ANOVA of Mosquito Net use and Parasite Density of Children Cohort**

**Table 6: Ordinary ONE-WAY ANOVA of Mosquito Repellent use and Parasite Density of Children Cohort**

| | |
|---|---|
| Table Analyzed | Mosquito Repellent _ Parasite density |
| ANOVA summary | |
| F | 2.137 |
| P value | 0.1201 |
| P value summary | ns |
| Are differences among means statistically significant? (P < 0.05) | No |
| R square | 0.01694 |
| Brown-Forsythe test | |
| F (DFn, DFd) | 4.452 (2, 248) |
| P value | 0.0126 |
| P value summary | * |
| Significantly different standard deviations? (P < 0.05) | Yes |
| Bartlett's test | |
| Bartlett's statistic (corrected) | 12.27 |

| | |
|---|---|
| P value | 0.0022 |
| P value summary | ** |
| Significantly different standard deviations? (P < 0.05) | Yes |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 4.88E+06 | 2 | 2.44E+06 | F (2 | 248) = 2.137 | P = 0.1201 |
| Residual (within columns) | 2.83E+08 | 248 | 1.14E+06 | | | |
| Total | 2.88E+08 | 250 | | | | |

| Data summary | |
|---|---|
| Number of treatments (columns) | 3 |
| Number of values (total) | 251 |

**Table 7: TWO-WAY ANOVA of Mosquito Net use and Highest Level of Education of Mothers in Children Cohort**

| | |
|---|---|
| Table Analyzed | Use of Mosquito Nets_ HLE of mothers_Children samples |
| Two-way ANOVA | Ordinary |
| Alpha | 0.05 |

| Source of Variation | % of total variation | P value | P value summary | Significant? |
|---|---|---|---|---|
| Row Factor | 76.08 | 0.0185 | * | Yes |
| Column Factor | 11.94 | 0.2508 | ns | No |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Row Factor | 3268 | 2 | 1634 | F (2 | 4) = 12.70 | P = 0.0185 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Column Factor | 512.9 | 2 | 256.4 | F (2 | 4) = 1.994 | P = 0.2508 |
| Residual | 514.4 | 4 | 128.6 | | | |

| | |
|---|---|
| Number of missing values | 0 |

**Table 8: Descriptive Analysis of Parasite density with respect to Age in Pregnant Women Cohort**

| *19 - 25 yr* | | *26 - 35 yr* | | *36 - 59 yr* | |
|---|---|---|---|---|---|
| Mean | 2041.143 | Mean | 1881.07 | Mean | 1855.294 |
| Standard Error | 202.2095 | Standard Error | 75.80845 | Standard Error | 136.3842 |
| Median | 1760 | Median | 1680 | Median | 1600 |
| Mode | 1760 | Mode | 1680 | Mode | 1280 |
| Standard Deviation | 1196.288 | Standard Deviation | 1036.665 | Standard Deviation | 973.9781 |
| Sample Variance | 1431105 | Sample Variance | 1074674 | Sample Variance | 948633.4 |
| Kurtosis | 1.518921 | Kurtosis | 0.120038 | Kurtosis | 3.34341 |
| Skewness | 1.262458 | Skewness | 0.811012 | Skewness | 1.441284 |
| Range | 5120 | Range | 4960 | Range | 4800 |
| Minimum | 480 | Minimum | 240 | Minimum | 320 |
| Maximum | 5600 | Maximum | 5200 | Maximum | 5120 |
| Sum | 71440 | Sum | 351760 | Sum | 94620 |
| Count | 35 | Count | 187 | Count | 51 |

**Table 9: Descriptive Analysis of Parasite density with respect to Mosquito Net use in Pregnant Women Cohort**

| *Always* | | *Some times* | | *Never* | |
|---|---|---|---|---|---|
| Mean | 1914.203 | Mean | 1973.333 | Mean | 1872.597 |
| Standard Error | 123.9126 | Standard Error | 165.2165 | Standard Error | 87.79678 |
| Median | 1760 | Median | 1840 | Median | 1560 |
| Mode | 1040 | Mode | 2000 | Mode | 960 |
| Standard Deviation | 1029.295 | Standard Deviation | 991.2993 | Standard Deviation | 1089.529 |
| Sample Variance | 1059448 | Sample Variance | 982674.3 | Sample Variance | 1187074 |
| Kurtosis | 2.018063 | Kurtosis | -0.19786 | Kurtosis | 0.584489 |
| Skewness | 1.17174 | Skewness | 0.548843 | Skewness | 0.991614 |
| Range | 4960 | Range | 4000 | Range | 5360 |
| Minimum | 240 | Minimum | 400 | Minimum | 240 |
| Maximum | 5200 | Maximum | 4400 | Maximum | 5600 |
| Sum | 132080 | Sum | 71040 | Sum | 288380 |
| Count | 69 | Count | 36 | Count | 154 |

**Table 10: Descriptive Analysis of Parasite density with to use of Mosquito Repellent in Pregnant Women Cohort**

| *Always* | | *Never* | | *Some times* | |
|---|---|---|---|---|---|
| Mean | 1986 | Mean | 1842.722 | Mean | 2012.8 |
| Standard Error | 171.626 | Standard Error | 83.2624 | Standard Error | 134.1845 |
| Median | 1840 | Median | 1600 | Median | 1760 |
| Mode | 1120 | Mode | 1760 | Mode | 1680 |
| Standard Deviation | 1085.458 | Standard Deviation | 1082.411 | Standard Deviation | 948.8275 |
| Sample Variance | 1178219 | Sample Variance | 1171614 | Sample Variance | 900273.6 |
| Kurtosis | -0.26931 | Kurtosis | 1.312124 | Kurtosis | -0.0014 |
| Skewness | 0.62227 | Skewness | 1.176979 | Skewness | 0.525908 |
| Range | 4560 | Range | 5360 | Range | 4080 |
| Minimum | 240 | Minimum | 240 | Minimum | 320 |
| Maximum | 4800 | Maximum | 5600 | Maximum | 4400 |
| Sum | 79440 | Sum | 311420 | Sum | 100640 |
| Count | 40 | Count | 169 | Count | 50 |

**Table 11: Descriptive Analysis of Parasite density with Trimesters in Pregnant Women Cohort**

| First | | Second | | Third | |
|---|---|---|---|---|---|
| Mean | 1558 | Mean | 1990 | Mean | 1939.389 |
| Standard Error | 133.7732 | Standard Error | 118.8226 | Standard Error | 92.83705 |
| Median | 1520 | Median | 1760 | Median | 1760 |
| Mode | 1040 | Mode | 960 | Mode | 1760 |
| Standard Deviation | 846.0563 | Standard Deviation | 1114.655 | Standard Deviation | 1062.569 |
| Sample Variance | 715811.3 | Sample Variance | 1242455 | Sample Variance | 1129052 |
| Kurtosis | 2.644292 | Kurtosis | 0.517764 | Kurtosis | 0.667169 |
| Skewness | 1.268948 | Skewness | 0.871644 | Skewness | 0.937413 |
| Range | 4160 | Range | 4880 | Range | 5280 |
| Minimum | 240 | Minimum | 240 | Minimum | 320 |
| Maximum | 4400 | Maximum | 5120 | Maximum | 5600 |
| Sum | 62320 | Sum | 175120 | Sum | 254060 |
| Count | 40 | Count | 88 | Count | 131 |

**Table 12: Ordinary ONE-WAY ANOVA of Parasite Density with respect to Age in Pregnant women**

| | | | |
|---|---|---|---|
| Table Analyzed | Parasite density_Age | | |
| ANOVA summary | | | |
| F | 0.3938 | | |
| P value | 0.6749 | | |
| P value summary | ns | | |
| Are differences among means statistically significant? (P < 0.05) | No | | |
| R square | 0.002908 | | |
| Brown-Forsythe test | | | |
| F (DFn | DFd) | 0.5450 (2 | 270) |
| P value | 0.5805 | | |
| P value summary | ns | | |
| Significantly different standard deviations? (P < 0.05) | No | | |
| Bartlett's test | | | |
| Bartlett's statistic (corrected) | 1.842 | | |
| P value | 0.3981 | | |
| P value summary | ns | | |

| | |
|---|---|
| Significantly different standard deviations? (P < 0.05) | No |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 863352 | 2 | 431676 | F (2 | 270) = 0.3938 | P = 0.6749 |
| Residual (within columns) | 2.96E+08 | 270 | 1.10E+06 | | | |
| Total | 2.97E+08 | 272 | | | | |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 3 |
| Number of values (total) | 273 |

**Table 13: Ordinary ONE-WAY ANOVA of Parasite Density with respect to Mosquito Net use in Pregnant women**

| | |
|---|---|
| Table Analyzed | Parasite density_Mosquito net |

ANOVA summary

| | |
|---|---|
| F | 0.143 |
| P value | 0.8668 |
| P value summary | ns |
| Are differences among means statistically significant? (P < 0.05) | No |
| R square | 0.001116 |

Brown-Forsythe test

| | | | |
|---|---|---|---|
| F (DFn | DFd) | 0.2570 (2 | 256) |

| | |
|---|---|
| P value | 0.7736 |
| P value summary | ns |
| Significantly different standard deviations? (P < 0.05) | No |

Bartlett's test

| | |
|---|---|
| Bartlett's statistic (corrected) | 0.6461 |
| P value | 0.724 |
| P value summary | ns |
| Significantly different standard deviations? (P < 0.05) | No |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 321768 | 2 | 160884 | F (2 | 256) = 0.1430 | P = 0.8668 |
| Residual (within columns) | 2.88E+08 | 256 | 1.13E+06 | | | |
| Total | 2.88E+08 | 258 | | | | |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 3 |
| Number of values (total) | 259 |

**Table 14: Ordinary ONE-WAY ANOVA of Parasite Density with respect to Mosquito Reppellent use in Pregnant women**

| | |
|---|---|
| Table Analyzed | Parasite density_Repellant |

ANOVA summary

| | |
|---|---|
| F | 0.6626 |
| P value | 0.5164 |
| P value summary | ns |

| | |
|---|---|
| Are differences among means statistically significant? (P < 0.05) | No |
| R square | 0.00515 |

Brown-Forsythe test

| | | | |
|---|---|---|---|
| F (DFn | DFd) | 0.3734 (2 | 256) |
| P value | 0.6887 | | |
| P value summary | ns | | |
| Significantly different standard deviations? (P < 0.05) | No | | |

Bartlett's test

| | |
|---|---|
| Bartlett's statistic (corrected) | 1.301 |
| P value | 0.5217 |
| P value summary | ns |
| Significantly different standard deviations? (P < 0.05) | No |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 1.49E+06 | 2 | 742547 | F (2 | 256) = 0.6626 | P = 0.5164 |
| Residual (within columns) | 2.87E+08 | 256 | 1.12E+06 | | | |
| Total | 2.88E+08 | 258 | | | | |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 3 |
| Number of values (total) | 259 |

**Table 15: Ordinary ONE-WAY ANOVA of Parasite Density with respect Trimesters in Pregnant women Cohort**

| | | | | | | |
|---|---|---|---|---|---|---|
| Table Analyzed | Parasite density_Trimesters | | | | | |
| ANOVA summary | | | | | | |
| F | 5.728 | | | | | |
| P value | < 0.0001 | | | | | |
| P value summary | **** | | | | | |
| Are differences among means statistically significant? (P < 0.05) | Yes | | | | | |
| R square | 0.08933 | | | | | |
| Brown-Forsythe test | | | | | | |
| F (DFn | DFd) | 5.833 (5 | 292) | | | |
| P value | < 0.0001 | | | | | |
| P value summary | **** | | | | | |
| Significantly different standard deviations? (P < 0.05) | Yes | | | | | |
| Bartlett's test | | | | | | |
| Bartlett's statistic (corrected) | 2234 | | | | | |
| P value | < 0.0001 | | | | | |
| P value summary | **** | | | | | |
| Significantly different standard deviations? (P < 0.05) | Yes | | | | | |
| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
| Treatment (between columns) | 3.01E+1 | 5 | 6.03E+1 | F (5 | 292) | P < |

| | | | | | |
|---|---|---|---|---|---|
| | 1 | | 0 | = 5.728 | 0.0001 |
| Residual (within columns) | 3.07E+12 | 292 | 1.05E+10 | | |
| Total | 3.37E+12 | 297 | | | |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 6 |
| Number of values (total) | 298 |

| *Always* | | *Sometimes* | | *Never* | |
|---|---|---|---|---|---|
| Mean | 1158.666667 | Mean | 1137.777778 | Mean | 1252.991 |
| Standard Error | 83.78593475 | Standard Error | 185.4927294 | Standard Error | 50.10866 |
| Median | 1040 | Median | 840 | Median | 1040 |
| Mode | 1200 | Mode | 800 | Mode | 800 |
| Standard Deviation | 649.0030599 | Standard Deviation | 786.9790008 | Standard Deviation | 766.5152 |
| Sample Variance | 421204.9718 | Sample Variance | 619335.9477 | Sample Variance | 587545.5 |
| Kurtosis | 1.765681714 | Kurtosis | 7.68370189 | Kurtosis | 1.50783 |
| Skewness | 1.340715822 | Skewness | 2.747759143 | Skewness | 1.216105 |
| Range | 2880 | Range | 3280 | Range | 3920 |
| Minimum | 240 | Minimum | 480 | Minimum | 160 |
| Maximum | 3120 | Maximum | 3760 | Maximum | 4080 |
| Sum | 69520 | Sum | 20480 | Sum | 293200 |

| | | | | | |
|---|---|---|---|---|---|
| Count | 60 | Count | 18 | Count | 234 |

**Table 16: Descriptive Analysis of Parasiste density with respect to use of mosquito nets in the Adult cohorts**

**Table 17: Descriptive Analysis of Parasiste density with respect to use of mosquito reppellents in the Adult cohorts**

| Always | | Sometimes | | Never | |
|---|---|---|---|---|---|
| Mean | 1100.488 | Mean | 1196 | Mean | 1288.477 |
| Standard Error | 87.71486 | Standard Error | 69.50710824 | Standard Error | 63.00396 |
| Median | 1040 | Median | 960 | Median | 1040 |
| Mode | 880 | Mode | 800 | Mode | 1200 |
| Standard Deviation | 561.6491 | Standard Deviation | 761.4122217 | Standard Deviation | 774.2056 |
| Sample Variance | 315449.8 | Sample Variance | 579748.5714 | Sample Variance | 599394.3 |
| Kurtosis | -0.0334 | Kurtosis | 2.080370684 | Kurtosis | 1.493763 |
| Skewness | 0.678486 | Skewness | 1.419273868 | Skewness | 1.269315 |
| Range | 2320 | Range | 3920 | Range | 3920 |
| Minimum | 160 | Minimum | 160 | Minimum | 160 |
| Maximum | 2480 | Maximum | 4080 | Maximum | 4080 |
| Sum | 45120 | Sum | 143520 | Sum | 194560 |
| Count | 41 | Count | 120 | Count | 151 |

**Table 18: Descriptive Analysis of Parasiste density with respect to Age in the Adult cohorts**

| *10 -25* | | *26 - 40* | | *41 - 55* | | *56 - 83* | |
|---|---|---|---|---|---|---|---|
| Mean | 1481.111 | Mean | 1174.468 | Mean | 1052.075 | Mean | 1200 |
| Standard Error | 98.43842 | Standard Error | 58.66364 | Standard Error | 87.44451 | Standard Error | 115.5169 |
| Median | 1240 | Median | 1040 | Median | 880 | Median | 1040 |
| Mode | 1200 | Mode | 800 | Mode | 960 | Mode | 1200 |
| Standard Deviation | 835.2777 | Standard Deviation | 696.5921 | Standard Deviation | 636.6057 | Standard Deviation | 783.4737 |
| Sample Variance | 697688.9 | Sample Variance | 485240.6 | Sample Variance | 405266.8 | Sample Variance | 613831.1 |
| Kurtosis | -0.02201 | Kurtosis | 3.782494 | Kurtosis | 2.806408 | Kurtosis | 1.620588 |
| Skewness | 0.801301 | Skewness | 1.603936 | Skewness | 1.577178 | Skewness | 1.369011 |
| Range | 3520 | Range | 3920 | Range | 2880 | Range | 3280 |
| Minimum | 240 | Minimum | 160 | Minimum | 160 | Minimum | 240 |
| Maximum | 3760 | Maximum | 4080 | Maximum | 3040 | Maximum | 3520 |
| Sum | 106640 | Sum | 165600 | Sum | 55760 | Sum | 55200 |
| Count | 72 | Count | 141 | Count | 53 | Count | 46 |

**Table 19: Ordinary ONE-WAY ANOVA of Parasite Density with Respect to Mosquito net use in the Adult Cohort**

| | | | |
|---|---|---|---|
| Table Analyzed | Mosquito net_Parasite density redo | | |
| ANOVA summary | | | |
| F | 0.5211 | | |
| P value | 0.5944 | | |
| P value summary | ns | | |
| Are differences among means statistically significant? (P < 0.05) | No | | |
| R square | 0.003362 | | |
| Brown-Forsythe test | | | |
| F (DFn | DFd) | 1.552 (2 | 309) |
| P value | 0.2134 | | |
| P value summary | ns | | |
| Significantly different standard deviations? (P < 0.05) | No | | |
| Bartlett's test | | | |
| Bartlett's statistic (corrected) | 2.508 | | |

| | |
|---|---|
| P value | 0.2853 |
| P value summary | ns |
| Significantly different standard deviations? (P < 0.05) | No |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 581084 | 2 | 290542 | F (2 | 309) = 0.5211 | P = 0.5944 |
| Residual (within columns) | 1.72E+08 | 309 | 557534 | | | |
| Total | 1.73E+08 | 311 | | | | |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 3 |
| Number of values (total) | 312 |

**Table 20: Ordinary ONE-WAY ANOVA of Parasite Density with respect to Age in the Adult Cohort**

| | |
|---|---|
| Table Analyzed | Age_Parasite density redo |

ANOVA summary

| | |
|---|---|
| F | 4.135 |
| P value | 0.0068 |
| P value summary | ** |
| Are differences among means statistically significant? (P < 0.05) | Yes |

| | |
|---|---|
| R square | 0.03872 |

Brown-Forsythe test

| | |
|---|---|
| F (DFn | DFd) 2.035 (3 308) |
| P value | 0.1089 |
| P value summary | ns |
| Significantly different standard deviations? (P < 0.05) | No |

Bartlett's test

| | |
|---|---|
| Bartlett's statistic (corrected) | 5.615 |
| P value | 0.1319 |
| P value summary | ns |
| Significantly different standard deviations? (P < 0.05) | No |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 6.69E+06 | 3 | 2.23E+06 | F (3 | 308) = 4.135 | P = 0.0068 |
| Residual (within columns) | 1.66E+08 | 308 | 539500 | | | |
| Total | 1.73E+08 | 311 | | | | |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 4 |
| Number of values (total) | 312 |

**Table 21: Ordinary ONE-WAY ANOVA of Parasite Density with respect to Repellent use in the Adult Cohort**

| | |
|---|---|
| Table Analyzed | Repellent_Parasite Density |

ANOVA summary

| | |
|---|---|
| F | 1.209 |
| P value | 0.3 |
| P value summary | ns |
| Are differences among means statistically significant? (P < 0.05) | No |
| R square | 0.007762 |

Brown-Forsythe test

| | |
|---|---|
| F (DFn DFd) | 0.6847 (2 309) |
| P value | 0.505 |
| P value summary | ns |
| Significantly different standard deviations? (P < 0.05) | No |

Bartlett's test

| | |
|---|---|
| Bartlett's statistic (corrected) | 5.868 |
| P value | 0.0532 |
| P value summary | ns |
| Significantly different standard deviations? (P < 0.05) | No |

| ANOVA table | SS | DF | MS | F (DFn DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 1.34E+06 | 2 | 670888 | F (2 309) = 1.209 | P = 0.3000 |
| Residual (within columns) | 1.72E+08 | 309 | 555072 | | |

| | | |
|---|---|---|
| Total | 1.73E+08 | 311 |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 3 |
| Number of values (total) | 312 |

**Table 22: TWO-WAY ANOVA of Mosquito Net use and Highest Level of Education of Mothers in the Adult Cohort**

| | |
|---|---|
| Table Analyzed | HLE_MOSQUITO NET USE |
| Two-way ANOVA | Ordinary |
| Alpha | 0.05 |

| Source of Variation | % of total variation | P value | P value summary | Significant? |
|---|---|---|---|---|
| Row Factor | 77.8 | 0.0149 | * | Yes |
| Column Factor | 11.37 | 0.2379 | ns | No |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Row Factor | 928.3 | 2 | 464.1 | F (2 | 4) = 14.37 | P = 0.0149 |
| Column Factor | 135.7 | 2 | 67.86 | F (2 | 4) = 2.101 | P = 0.2379 |
| Residual | 129.2 | 4 | 32.3 | | | |

| | |
|---|---|
| Number of missing values | 0 |

**Table 23: Decriptive Statistics for the Parasite Density from the three cohorts**

| *Mixed Cohort* | | *Children Cohort* | | *Preg women Cohort* | |
|---|---|---|---|---|---|
| Mean | 1205.513 | Mean | 2107.6 | Mean | 1897.683 |
| Standard Error | 41.9193 | Standard Error | 66.79542 | Standard Error | 65.69359 |
| Median | 1040 | Median | 1760 | Median | 1680 |
| Mode | 800 | Mode | 1200 | Mode | 1760 |
| Standard Deviation | 740.4425 | Standard Deviation | 1056.128 | Standard Deviation | 1057.238 |
| Sample Variance | 548255 | Sample Variance | 1115407 | Sample Variance | 1117753 |
| Kurtosis | 2.773668 | Kurtosis | 1.344696 | Kurtosis | 0.765011 |
| Skewness | 1.522666 | Skewness | 1.151212 | Skewness | 0.969941 |
| Range | 4080 | Range | 5920 | Range | 5360 |
| Minimum | 80 | Minimum | 320 | Minimum | 240 |
| Maximum | 4160 | Maximum | 6240 | Maximum | 5600 |
| Sum | 376120 | Sum | 526900 | Sum | 491500 |
| Count | 312 | Count | 250 | Count | 259 |

| | |
|---|---|
| Table Analyzed | Parasite density_Mosquito net use_All cohorts |

ANOVA summary

| | |
|---|---|
| F | 1.265 |
| P value | 0.2829 |
| P value summary | ns |
| Are differences among means statistically significant? (P < 0.05) | No |
| R square | 0.003083 |

Brown-Forsythe test

| | | | |
|---|---|---|---|
| F (DFn | DFd) | 0.1856 (2 | 818) |
| P value | 0.8307 | | |
| P value summary | ns | | |
| Significantly different standard deviations? (P < 0.05) | No | | |

Bartlett's test

| | |
|---|---|
| Bartlett's statistic (corrected) | 0.9146 |
| P value | 0.633 |
| P value summary | ns |
| Significantly different standard deviations? (P < 0.05) | No |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 2.67E+06 | 2 | 1.33E+06 | F (2 | 818) = 1.265 | P = 0.2829 |
| Residual (within columns) | 8.62E+08 | 818 | 1.05E+06 | | | |
| Total | 8.65E+08 | 820 | | | | |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 3 |
| Number of values (total) | 821 |

**Table 24: Ordinary ONE-WAY ANOVA of Parasite Density with respect to three cohorts sampled.**

**Table 25: Decriptive Statistics for the Parasite Density with respect to Mosquito Net use**

| *Always* | | *Never* | | *Sometimes* | |
|---|---|---|---|---|---|
| Mean | 1773.581 | Mean | 1502.051 | Mean | 1788.657 |
| Standard Error | 72.42662 | Standard Error | 56.36664 | Standard Error | 128.6265 |
| Median | 1600 | Median | 1240 | Median | 1600 |
| Mode | 1200 | Mode | 960 | Mode | 800 |
| Standard Deviation | 1061.983 | Standard Deviation | 995.6334 | Standard Deviation | 1052.853 |
| Sample Variance | 1127807 | Sample Variance | 991285.8 | Sample Variance | 1108500 |
| Kurtosis | 2.049131 | Kurtosis | 1.685821 | Kurtosis | 0.383673 |
| Skewness | 1.36878 | Skewness | 1.332483 | Skewness | 1.015309 |
| Range | 5520 | Range | 5520 | Range | 4480 |
| Minimum | 240 | Minimum | 80 | Minimum | 400 |
| Maximum | 5760 | Maximum | 5600 | Maximum | 4880 |
| Sum | 381320 | Sum | 468640 | Sum | 119840 |
| Count | 215 | Count | 312 | Count | 67 |

**Table 26: Decriptive Statistics for the Parasite Density with respect to use of Mosquito Repellent**

| *Always* | | *Never* | | *Sometimes* | |
|---|---|---|---|---|---|
| Mean | 1919.685 | Mean | 1666.923 | Mean | 1573.592 |
| Standard Error | 107.4926 | Standard Error | 59.5986 | Standard Error | 65.35246 |
| Median | 1600 | Median | 1360 | Median | 1360 |
| Mode | 960 | Mode | 1200 | Mode | 800 |
| Standard Deviation | 1211.38 | Standard Deviation | 1052.721 | Standard Deviation | 937.9842 |
| Sample Variance | 1467441 | Sample Variance | 1108222 | Sample Variance | 879814.3 |
| Kurtosis | 1.376537 | Kurtosis | 1.879438 | Kurtosis | 0.260905 |
| Skewness | 1.224815 | Skewness | 1.375065 | Skewness | 0.863564 |
| Range | 6000 | Range | 5600 | Range | 4240 |
| Minimum | 240 | Minimum | 80 | Minimum | 160 |
| Maximum | 6240 | Maximum | 5680 | Maximum | 4400 |
| Sum | 243800 | Sum | 520080 | Sum | 324160 |
| Count | 127 | Count | 312 | Count | 206 |

| | |
|---|---|
| Table Analyzed | Parasite density_Mosquito net use_All cohorts |

ANOVA summary

| | |
|---|---|
| F | 1.265 |
| P value | 0.2829 |
| P value summary | ns |
| Are differences among means statistically significant? (P < 0.05) | No |
| R square | 0.003083 |

Brown-Forsythe test

| | | | |
|---|---|---|---|
| F (DFn | DFd) | 0.1856 (2 | 818) |
| P value | 0.8307 | | |
| P value summary | ns | | |
| Significantly different standard deviations? (P < 0.05) | No | | |

Bartlett's test

| | |
|---|---|
| Bartlett's statistic (corrected) | 0.9146 |
| P value | 0.633 |
| P value summary | ns |
| Significantly different standard deviations? (P < 0.05) | No |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 2.67E+06 | 2 | 1.33E+06 | F (2 | 818) = 1.265 | P = 0.2829 |
| Residual (within columns) | 8.62E+08 | 818 | 1.05E+06 | | | |
| Total | 8.65E+08 | 820 | | | | |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 3 |
| Number of values (total) | 821 |

**Table 27: Ordinary ONE-WAY ANOVA of Parasite Density with respect to use of Mosquito nets in overall cohorts**

**Table 28: Ordinary ONE-WAY ANOVA of Parasite Density with respect to use of Mosquito Repellents in overall cohorts**

| | |
|---|---|
| Table Analyzed | Parasite density_Repellants_All cohorts |

ANOVA summary

| | |
|---|---|
| F | 4.514 |
| P value | 0.0112 |
| P value summary | * |
| Are differences among means statistically significant? (P < 0.05) | Yes |
| R square | 0.01092 |

Brown-Forsythe test

| | | | |
|---|---|---|---|
| F (DFn | DFd) | 3.455 (2 | 818) |
| P value | 0.032 | | |
| P value summary | * | | |
| Significantly different standard deviations? (P < 0.05) | Yes | | |

Bartlett's test

| | |
|---|---|
| Bartlett's statistic (corrected) | 11.35 |
| P value | 0.0034 |
| P value summary | ** |

| | |
|---|---|
| Significantly different standard deviations? (P < 0.05) | Yes |

| ANOVA table | SS | DF | MS | F (DFn | DFd) | P value |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 9.44E+06 | 2 | 4.72E+06 | F (2 | 818) = 4.514 | P = 0.0112 |
| Residual (within columns) | 8.55E+08 | 818 | 1.05E+06 | | | |
| Total | 8.65E+08 | 820 | | | | |

Data summary

| | |
|---|---|
| Number of treatments (columns) | 3 |
| Number of values (total) | 821 |

**Table 29: Turkey's Multiple Comparison Tests for the Parasite densities of the three cohorts sampled**

| | |
|---|---|
| Number of families | 1 |
| Number of comparisons per family | 3 |
| Alpha | 0.05 |

| Tukey's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary |
|---|---|---|---|---|
| Adult Cohort vs. Children Cohort | -902.1 | -1091 to -713.0 | Yes | **** |
| Adult Cohort vs. Preg women Cohort | -692. | -879.5 to - | Yes | **** |

| | | | | |
|---|---|---|---|---|
| | 2 | 504.9 | | |
| Children Cohort vs. Preg women Cohort | 209.9 | 12.37 to 407.5 | Yes | * |

| Test details | Mean 1 | Mean 2 | Mean Diff. | SE of diff. | n1 | n2 | q | DF |
|---|---|---|---|---|---|---|---|---|
| Adult Cohort vs. Children Cohort | 1206 | 2108 | -902.1 | 80.55 | 312 | 250 | 15.84 | 818 |
| Adult Cohort vs. Preg women Cohort | 1206 | 1898 | -692.2 | 79.77 | 312 | 259 | 12.27 | 818 |
| Children Cohort vs. Preg women Cohort | 2108 | 1898 | 209.9 | 84.14 | 250 | 259 | 3.528 | 818 |

**Table 29: Sample sequence (forward) of *Pfcrt* from children cohort**

CGTGCTGTATAGTGCTCATGTTGTTTAAACTTATTTTTAAAGAGATTAAG
GATAATATTTTTATTTATATTTTAAGTATTTATTTATTTAAGTGTATGTG
TAATTGAAACAATTTTTGCTAAAAGAACTTTAAACAAAATTGGTAACTAT
AGTTTTGTAACATCCGAAACTCACAACTTTATTTGTATGATTATGTTCTT
TATTGTTTATTCCTTATTTGGAAATAAAAAGGGAAATTCAAAGTAAGTAA
GATAAATCAATATATTAAAATGATGGATTTATAAGAGAATCTATTCCACC
TAAACGTGAGCCA

**Table 30: Sample sequence (forward) of *Pfmdr1 (codon 86)* from children cohort**

TTGATAAGATGAGAAATATCATTTTTTTTACCGTTTAAATGTTTACCTGC

ACAACATAGAAAATTATTATTTATATCATTTGTATGTGCTGTATTATCAG
GAGGAACATTACCTTTTTTTATATCTGTGTTTGGTGTAATATTAAAGAAC
ATGAATTTAGGTGATGATATTAATCCTATAATATTATCATTAGTATCTAT
AGGTTTAGTACAATTTATATTATCAATGATATCAAGTTATTGTATGGATG
TAATTACATCAAAAATATTAAAAACTTTAAAGCTTGAATATTTAAGAAGT
GTTTTTTATCAAGATGGACAATTTCATGATAATAATCCTGGATCTAAATT
AAGATCTGATTTAGATTTTTATTTAGAACAAGTGAGTTCAGGAATTGGTA
CGAAATTTATAACAATTTTTACATATGCCAGTTCCTTTTTAGGTTTATAT
ATTTGGTCATTAATAAAAAATGCACGTTTGACTTTATGTATTACTTGC

**Table 31: Sample sequence (forward) of *Pfmdr1 (codon 1034)* from children cohort**

CATTTTTTTATACAGGAAGCATTTTATAATATGCATACTGTTATTAATTA
TGGTTTAGAAGATTATTTCTGTAATTTGATAGAAAAAGCTATTGATTATA
AAAATAAAGGACAAAAAAGAAGAATTATTGTAAATGCAGCTTTATGGGGA
TTCAGTCAAAGCGCTCAATTATTTATTAATAGTTTTGCCTATTGGTTTGG
ATCCTTCTTAATTAAAAGAGGTACTATATTAGTTGATGACTTTATGAAAT
CCTTATTTACTTTTATATTTACTGGTAGTTATGCTGGAAAATTAATGTCC
TTAAAAGGAGATTCAGAAAATGCAAAATTATCATTTGAGAAATATTATCC
ATTAATGATTAGAAAATCAAATATTGATGTAAGAGATGATGGTGGAATAA
GAATAAATAAAAATTTAATAAAAGGTAAAGTTGATATTAAAGATGTAAAT
TTCCGTTATATTTCAAGACCAAATGTACCTATTTATAAAAATTTATCTTT
TACATGTGATAGTAAAAAAACTACAGCAATCGTTGGAGAAACAGGTAGTG
GAAAATCAACTTTTATGAATCTCTTATTAAGATTTTATGACTTGAAAAAT
GATCACATTATATTAAAAAATGATATGACAAATTTTCAAGATTATCAAAA
TAATAATAATAATTCATTGGTTTTAAAAAATGTAAATGAATTTTCAAACC
AATCTGGATCTGCAGAAGATTATACTGTATTTAATAATAATGGAGAAATA
TTATTAGATGATATTAATATATGTGATTATAACTTAAGAGATCTTAGAAA
CTTATTTTCAATAGTTAGTCAAGACCATTTTTTTTATGTTTAAAAATATA
TACACGCCAAGGTGCAGAATATTCCTCGCGCTGTGGAACAATTATTATAT
ACATCACGTGCGGAATCTCTGCGTTACCTTTGGGATAAACCCCAAGCCTC
TTACAGCAAATATCACACACA

**Table 32: Sample sequence (Reverse) of *Pfmdr1 (codon 1034)* from children cohort**

AGTGACAAGACCTCTAAGATCTCTTAAGCCATAATCCATATATTAATATC
ATCTAATAATATCCCTCCCCATCATCAAACACAGCATAATCCCCCGCAGA
TCCAGATCGGCCCGAAAACCCATCCACACCCTTTAAAACCAATGAACCAT
CATCATTATCTTGATAATCTTGAAAATCTGTCATATCATCTTTTAATATA
ATGTGATCATCCTTCAAGCCATAAAATCTTAATAAGAGATTCATAAAAGT
TGATTTTCCACTACCTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTAT
CACATGTAAAAGATAAATTTTTATAAATAGGTACATTTGGTCTTGAAATA
TAACGGAAATTTACATCTTTAATATCAACTTTACCTTTTATTAAATTTTT
ATTTATTCTTATTCCACCATCATCTCTTACATCAATATTTGATTTTCTAA
TCATTAATGGATAATATTTCTCAAATGATAATTTTGCATTTTCTGAATCT

CCTTTTAAGGACATTAATTTTCCAGCATAACTACCAGTAAATATAAAAGT
AAATAAGGATTTCATAAAGTCATCAACTAATATAGTACCTCTTTTAATTA
AGAAGGATCCAAACCAATAGGCAAAACTATTAATAAATAATTGAGCGCTT
TGACTGAATCCCCATAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCC
TTTATTTTTATAATCAATAGCTTTTTCTATCAAATTACAGAAATAATCTT
CTAAACCATAATTAATAACAGTATGCATATTATAAAATGCTTCCTGTATT
AAAAAACTTGGATCTTTAACATTCTCCCTCTGGGAGAAAATGGAACCACT
AAATTGCAAAACCTCCGCTTGACATATTTAAATTATCTTGTCATCCTGCT
TACTTTTCGAAAAACCCAAGCTTTTTACCGCAATTCCCCCACA

**Table 33: Sample Sequences (Forward) of Pfatpase (codon 241-431) from children Samples**

CACCCCTGTACCCTTTCTAACAAGACTACGTATGAAGACAAGAAAATAGG
CCACCCCTCAGCATGCTGTTATAGAATCAAATAGTGAAGATACTTCAAAC
ACCTTTACAAATAAAAATCGATTTATTTGGTCAACAATTATCAAAAATCA
TTTTTGTAATATGTGTAACTGTATGGATTATTAATTTTAAACATTTCTCA
GATCCAATTCATGGTTCATTTTTATATGGTTGTTTATATTATTTTAAAAT
TAGTGTTGCTTTAGCTGTTGCTGCTATACCAGAAGGATTGCCAGCTGTCA
TAACAACTTGTTTAGCTTTAGGAACAAGAAGAATGGTAAAAAAAAAATGCT
ATAGTAAGAAAATTACAAAGTGTTGAGACCTTAGGATGTACAACGGTTAT
ATGTTCTGATAAAACAGGTACCCTTACAACAAATTAAATGACAACAACCG
TGTTTCATTTGTTTAGAGAATCTGATTCTTTAACAGAATACCAACTATGT
CAAAAAGGGGATACCTATTACTTTTATGAAAGTCCAAACGTAACAAATGA
TATATTTGCAGGTGAATCATCTTTTTTTAATAAATTAAAAGATGAAGGAA
ATGTT

**Table 34: Sample Sequences (Forward) of *Pfatpase* (codon 241-431) from children Samples**

AATATCGGCGTAGATATGCTTTGGTATATTACAACATTCTCTAAAAATAA
TAAAATGCCAGCAGAATATGAAAAAAATACAACACCTGTACAATCATCAA
ATAAGAAGGATAAATCACCAAGGGGTATCAACAAATTCTTTAGTTCAAAA
AATGATAACAGTCATATTACCAGTACATTGAATGAAAATGATAAGAATTT
AAAGAATGCTAACCATTCTAATTATACTACAGCTCAGGCAACAACAAATG
GATATGAAGCTATAGGAGAAAATACATTTGAGCATGGCACAAGTTTTGAA
AATTGTTTCCACTCAAAATTGGGTAATAAAATAAATACCACATCAACACA
TAATAATAATAACAACAATAATAATAATAGTAATAGTGTTCCAAGTGAAT
GTATTTCTTCTTGGAGAAATGAATGTAAACAAATAAAAATTATTGAATTC
ACTAGAGAAAGGAAACTTATGAGTGTTATTGTTGAAAATAAAAAAAAAGA
AATAATATTGTATTGTAAAGGTGCACCTGAGAATATAATAAAAAATTGTA

AATATTATTTAACGAAAAATGATATACGTCCATTAAATGAAACTTTAAAA
AATGAAATTCATAATAAGATTCAAAATATGGGAAAAAGAGCATTAAGAAC
ACTTAGCTTTGCTTATAAAAAATTAAGTAGTAAAGATTTAAATATTAAGA
ATACAGATGATTATTATAAATTAGAACAAGATTTAATTTATTTAGGTGGA
TTAGGTATTATTGATCCACCACGTAAATATGTAGGGAAAGA

**Table 35: Sample Sequences (Forward) of *Pfkelch* 13 from children Samples**

CATGAGGATTAAATTTTTACCTTCCCATTAGTATTTTGTATAGGTGGATT
TGATGGTGTAGAATATTTAAATTCGATGGAATTATTAGATATTAGTCAAC
AATGCTGGCGTATGTGTACACCTATGTCTACCAAAAAAGCTTATTTTGGA
AGTGCTGTATTGAATAATTTCTTATACGTTTTTGGTGGTAATAACTATGA
TTATAAGGCTTTATTTGAAACTGAGGTGTATGATCGTTTAAGAGATGTAT
GGTATGTTTCAAGTAATTTAAATATACCTAGAAGAAATAATTGTGGTGTT
ACGTCAAATGGTAGAATTTATTGTATTGGGGGATATGATGGCTCTTCTAT
TATACCGAATGTAGAAGCATATGATCATCGTATGAAAGCATGGGTAGAGG
TGGCACCTTTGAATACCCCTAGATCATCAGCTATGTGTGTTGCTTTTGAT
AATAAAATTTATGTCATTGGTGGAACTAATGGTGAGAGATTAAATTCTAT
TGAAGTATATGAAGAAAAAATGAATAAATGGGAACAATTTCCATATGCCT
TATTAGAAGCTAGAAGTTCAGGAGCAGCTTTTAATTACCTTAATCAAATA
TATGTTGTTGGAGGTATTGATAATGAACATAACATATTAGATTCCGTTGA
ACAATATCAACCATTTAATAAAAGATGGCAATTTCTAAATGGTGTACCAG
AGAAAAAAATGAATTTTGGAGCTGCCACATTGTCAGATTCTTATATAATT
ACAGGAGGAGAAAATGGCGAAGTTCTAAATTCATGTCATTTCTTTTCACC
AGATACAAATGAATGGCACTTGGCA

**Table 36: Sample Sequences (Reverse) of *PfCSP* from children Samples**

ATGACTTTTACGACATTAAACACACTGGAACATTTTTCCATTTTACAAAT
TTTTTTTTCAATATCATTTTCATAATTTAATTCGTCTTTAGGTTTATTAG
CAGAGCCAGGCTTTATTCTAACTTGAATACCATTTCCACAAGTTACACTA
CATGGGGACCATTCAGTTGAAATAGAATTTTTTATTTTGTTTAAATATTG
TTCTATGTGCTTATCACTTGGTTCTTCGTTATTATTATTTTTTACAGCAT
TGTTGGCATTAGCATTTTCATCTACATTTCGGTTTGGGTCATTTGGCATA
TTGTGACCTTGTCCATTACCTTGATTGTTTTTATTAGGATTTGCATTGGG
GTTTGCATTTGGGTTTGCATTTGGGTTTGCATTTGGATTTGCATTTGGAT
TGGCATTAGGATTTGCATTGGGGTTTGCGTTTGGGTTTGCATTTGGATTT

GCATTTGGATTGGCATTAGGATTTGCATTAGGATTTGCATTGGGGTTTGC
ATTAGGATTTGCATTGGGGTTTGCGTTTGGGTTTGCATTTGGATTTGCAT
TAGGATCTACATTTGGGTTTGCATTTGGGTTTGCATTAGGATTTGCATTG
GGGTTTGCATTTGGGTTTGCATTTGGGTTTGCATTTGGGTTTGCATTTGG
GTTTGCATTTGGGTTTGCATTTGGGTTTGCATTTGGGTTTGCATTTGGGT
TTGCATTTGGGTTTGCATTTGGGTTTGCATTTGGGTTTGCATTTGGATCT
ACATTTGGGTTTGCATTTGGATCTACATTTGGGTTGGCATTGGGATCTAC
ATTTGGGTTTGCATTTGGATCAGGATTACCATCCGCTGGTTGCTTTAATT
TTTTATGTTTGGGTTTCCTAAATTCTCGTGTCTTCGTAATTTCCATCATC
ATTTTCTCCAGTGATCTACTACTATTTATAAAGAACTATACCAATTTCCC
GCTTGCCATAATATTCCATTACACC

**Table 37: Sample Sequences (Forward) of *PfCSP* from children Samples**

CGATTGGTATAGTCTTAAAAAAAATAGTAGATCCTTGGGAGAAAATGATG
ATGGAAATAACGAAGACAACGAGAAATTAAGGAAACCAAAACATAAAAAA
TTAAAGCAACCAGCGGATGGTAATCCTGATCCAAATGCAAACCCAAATGT
AGATCCCAATGCCAACCCAAATGTAGATCCAAATGCAAACCCAAATGTAG
ATCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAAC
CCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCC
AAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCTA
ATGCATATCCTAATGCAAACCCAATGCAAACCCTAATGCAAATCCTAATG
CAAATCCAATGCAAACCCAAATGCAAACCCCAATGCAAATCCTAATGCAA
ACCCCAATGCAAATCCTAATGCAAATCCTAATGCCAATCCAATGCAAATC
CAATGCAAACCCAAATGCAAACCCAATGCAAATCCAATGCCAATCCAATG
CAAATCCTAATGCAAACCCTAATGCAAACCCTAATGCAAACCCTAATGCA
AATCCTAATGAAAACAATCAAGGTAATGGACAATGTCACAATATGCCAAA
TGACCCAGACCAAAATGTTGATGATAATGCTGCTGCCAACAATGCTGTTT
AAAATGATAATACCGAAGAACCCGGTGATAAGCACATAGAACCATATTTA
AATACAATATAAAATTCTTTTTCACTGAATGGTCCCCATGTAGTGTTACT
TGTGGAGGGGGGTTTTATTATAAAATAACAGTGCTCCCTCCTCTCAAGAC
ACATAAATATAAAATTGCTGTGACAAGGACTGTTAGTGGAAGAGTGTTCA
TCGTGTCTATGGCCACCATAATCAAAAGATTTATAATAAGAATATCCTCC
CCCCACATTCT

**Table 37: Sample Sequences (Forward) of *P.vivax* from children Samples**

CAAGATGAGTGACGTGCTATCCGCTTGTCTAGCGGTCGTGGTTAAATAAT
GGTATCTTTTAATTAAAATGATTCTTTTTAAGGACTTTCTTTGCTTCGGC
TTGGAAGTA

**Table 38: Sample Sequences (Forward) of *Pcrt* from Pregnant women Samples**

TGTGTCTGTACTCTGCCTATGTCCCAAACCCATCCCCAAAGAGCCAAGGA
AACACCCCCACCCACACCCCAAGCACCACCCACCCAAGCGCACGCGCAAC
CGAAACAACCCCCGCCAAAAGAACCCCAAACAAAACCGGTAACCACAGCC
CCGTAACACCCGAAACCCACAACCCCATCCGTATGATCACGCCCCCCACC

GTCTACCCCTCATTTGGAAATAAAAAGGAAATTCAAAATAAGTAAGATAA
ATCAATATATTAAAATGATGGATTTATAAGAGAATCTATTCCACCTACCA
ATATAAAACATTACACATATTTTTTTTTTTTATATATATATATGTGTGT
GTGTGTTG

**Table 39: Sample Sequences (Reverse) of *Pcrt* from Pregnant women Samples**

TGGATGACATAAAGACATAATCTACAAATAAAGTCTGTGAGTTTTCGGAT
GTTACAAAACTATAGTTACCAATCTTTGTTTAAAGTTCTTTTAGCAAAAA
TTGTTTCAATTACACATACACTTAAATAAATAATACTTAAAATATAAATA
AAAATATTATCCTTAATCTCTTTAAAAATAAGTTTAAACACATGAGCACA
TTTACCAAGACAAGAACCTCCACCTAAACGTGAGCCATCTCTGTAAGTGT
CCAAAGGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATTTTAAAA
CTTGGGGGAAAACATTTTTTTTTTTTTTTTTTTTTTTTTTATCCCCGG
GGGTTTTTTTTTTTTTTTTTTTTTTTTATCAAAAAATCCCCTTTTTGGGGG
ACACACTTCTTTTTTTTAAAAAAAATTTTTTTTTTCCCCACGGGCCGCCC
C

**Table 40: Sample Sequences (Forward) of Pfmdr1 (codon 86) from Pregnant women Samples**

CAGAAATAAGACTGAGAAAATCTCCCCCCTACCGTCTAAACGCCCACCTG
CACAACATAGAAAACCACCACCCACACCATCCGTACGCGCCGAACCAACA
GGAGGAACAACACCACCCCCAAAAACAGAGCCAGGCGCAACACCAAAGAA
CACGCACCCAGGCGATGAAACCAACCCTACAAAACCATCATCAGCACCTA
CAGGCCCAGCACAACCCATACCATCAATGATATCAAGCCATTGTATGGAT
GCAATCACATCAAAAATATTAAAAACTCTAAAGCTTGAATATTTAAGAAG
TGTTTTTTATCAAGATGGACAATCTCATGATAATAATCCTGGATCTAAAT
TAAGATCTGATTTAGATTTTTATTTAGAACAAGTGAGTTCAGGAATTGGT
ACGAAATTTATAACAATTTTTACATATGCCAGTTCCTTTTTAGGTTTATT
TATTTGGTCATTAATAAAAAATGCACGTTTGACTTTATGTATTACTTGCC
GTTTTTCCCCGTTCCTTTATGTTTGTGGTGGTCATAATCAAA

**Table 41: Sample Sequences (Reward) of *Pfmdr1* (codon 86) from Pregnant women Samples**

AAATGCATTTTTTATTAATGACCAATAAATAAACCTAAAAAGGAACTGGC
ATATGTAAAAATCTGTTATAAATTTCGTACCAATCCCTGAACTCACTTGT
TCTAAATAAAAATCTAAATCAGATCTTAATCTAGATCCAGGATTATTATC
ATGAAATTGTCCATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTA
AAGTTTTTAATATTTTTGATGTAATTACATCCATACAATAACTTGATATC
ATTGATAATATAAATTGTACTAAACCTATAGATACTAATGATAATATTAT
AGGATTAATATCATCACCTAAATACATGTTCTTTAATATTACACCAAACA
CAGATATAAAAAAAGGTAATGTTCCTCCTGATAATACAGCACATACAAAT
GATATAAATAATAATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAA
AAAAAATGATATTTTCTCATTCTTTATTTTTCTAAATAATTCAGCGGTAC

TCTTTTTTTGTTACAACCATTTTTAAACCAAAC

**Table 42: Sample Sequences (Forward) of *Pfmdr1* (codon 1034) from Pregnant women Samples**

CGCCCTTTATACAGGAAGCATCTTTATAATATGCATACTGTTATTAATCA
TGGTCTAGAAGATTATTTCTGTAATTTGATAGAAAAAGCTATTGATTATA
AAAATAAAGGACAAAAAAGAAGAATTATTGTAAATGCAGCTTTATGGGGA
TTCAGTCAAAGCGCTCAATTATTTATTAATAGTTTTGCCTATTGGTTTGG
ATCCTTCTTAATTAAAAGAGGTACTATATTAGTTGATGACTTTATGAAAT
CCTTATTTACTTTTATATTTACTGGTAGTTATGCTGGAAAATTAATGTCC
TTAAAAGGAGATTCAGAAAATGCAAAATTATCATTTGAGAAATATTATCC
ATTAATGATTAGAAAATCAAATATTGATGTAAGAGATGATGGTGGAATAA
GAATAAATAAAAATTTAATAAAAGGTAAAGTTGATATTAAAGATGTAAAT
TTCCGTTATATTTCAAGACCAAATGTACCTATTTATAAAAATTTATCTTT
TACATGTGATAGTAAAAAAACTACAGCAATCGTTGGAGAAACAGGTAGTG
GAAAATCAACTTTTATGAATCTCTTATTAAGATTTTATGACTTGAAAAAT
GATCACATTATATTAAAAAATGATATGACAAATTTTCAAGATTATCAAAA
TAATAATAATAATTCATTGGTTTTAAAAAATGTAAATGAATTTTCAAACC
AATCTGGATCTGCAGAAGATTATACTGTATTTAATAATAATGGAGAAATA
TTATTAGATGATATTAATATATGTGATTATAAACTTATGAGATCTTAGAA
ACTTATTTTCAAAAAATATAAGAAAAAGAACCCTGATCAAGCTAAAAATA
GACGAACAAGATGCTCAGTCGCTGTATTCTGCGTGTAAGAACCTACGATC
AACTCGTCTGTTTATGTAGTTAGATCAAGAATCACACTGGTGTTCTTAAC
GCAGTATACCAGCAATCTGAAGATGATTAAGATAGCAGTGTTTCCTGCGA
CCGGAAGTATTAAATAAACAGTGGGCCTCCCGTCGCCCACACGGAGAAAT
GTCACACTGTGTGAGAGTAGTTCA

**Table 43: Sample Sequences (Reverse) of *Pfmdr1* (codon 1034) from Pregnant women Samples**

CGCTGATTCGTTTGTAACATCTCTTAAGTTATAATCACATATATTAATAT
CATCTAATAATATTTCTCCATTATTATTAAATACAGTATAATCTTCTGCA
GATCCAGATTGGTTTGAAAATTCATTTACATTTTTTAAAACCAATGAATT
ATTATTATTATTTTGATAATCTTGAAAATTTGTCATATCATTTTTTAATA
TAATGTGATCATTTTTCAAGTCATAAAATCTTAATAAGAGATTCATAAAA
GTTGATTTTCCACTACCTGTTTCTCCAACGATTGCTGTAGTTTTTTTACT
ATCACATGTAAAAGATAAATTTTTATAAATAGGTACATTTGGTCTTGAAA
TATAACGGAAATTTACATCTTTAATATCAACTTTACCTTTTATTAAATTT

TTATTTATTCTTATTCCACCATCATCTCTTACATCAATATTTGATTTTCT
AATCATTAATGGATAATATTTCTCAAATGATAATTTTGCATTTTCTGAAT
CTCCTTTTAAGGACATTAATTTTCCAGCATAACTACCAGTAAATATAAAA
GTAAATAAGGATTTCATAAAGTCATCAACTAATATAGTACCTCTTTTAAT
TAAGAAGGATCCAAACCAATAGGCAAAACTATTAATAAATAATTGAGCGC
TTTGACTGAATCCCCATAAAGCTGCATTTACAATAATTCTTCTTTTTTGT
CCTTTATTTTTATAATCAATAGCTTTTTCTATCAAATTACAGAAATAATC
TTCTAAACCATAATTAATAACAGTATGCATATTATAAAATGCTTCCTGTA
TTAAAAAACTTGGATCTTTAAACATTTCATACATCTGAAACAAAATACAC
TCAGTTGAGTATTTGCTGCTAAGACGCTTTTACCATTTTTGTGTACTGCG
GTTCCAGCCGGGAAGCATCCCGCTTCATAACAGGAGATGACAGTAGGTGG
ATGTGGTGGATGCCTGGTTTCCATGAGGCTGAAGTTTTT

**Table 44: Sample Sequences (Reverse) of *Pfdhfr* from Pregnant women Samples**

TGTTCGATTTATGCCTATGTTGCATGTTTGTAAGGTCTGAAAGCAAAAAT
GAGGGGAAAAAAAATGAGGTTTTTTTAATAACTACACATTTAGAGGTCTA
GGAAATAAAGGAGTATTACCATGGAAATGTATTTCCCTAGATATGAAATA
TTTTCGTGCAGTTACAACATATGTGAATGAATCAAAATATGAAAAATTGA
AATATAAGAGATGTAAATATTTAAACAAAGAAACTGTGGATAATGTAAAT
GATATGCCTAATTCTAAAAAATTACAAAATGTTGTAGTTATGGGAAGAAC
AAACTGGGAAAGCATTCCAAAAAAATTTAAACCTTTAAGCAATAGGATAA
ATGTTATATTGTCTAGAACCTTAAAAAAAGAAGATTTTGATGAAGATGTT
TATATCATTAACAAAGTTGAAGATCTAATAGTTTTACTTGGGAAATTAAA
TTACTATAAATGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTT
TAGAAAAGAAATTAATAAAAAAAATATATTTTACTAGAATAAATAGTACA
TATGAATGTGATGTATTTTTTCCAGAAATAAATGAAAATGAGTATCAAAT
TATTTCTGTTAGCGATGTATATCCAAAATCAAAGGAAAA

**Table 45: Sample Sequences (Reverse) of *Pfdhps (438)* from Pregnant women Samples**

TGTGCACACTAGCCCTGAACTGATAAACTGAAGGCTGCCAGCGCCACAGA
TGCAGGCGGAGAATCCGCCGGTCCTCCCGTCACACCCAATCCAAAAACCA
GCGAAAGAGATCCAGCAGCACCCGCACCACAACCACCCCAAAAAGAATGG
AATGATACAAAAAACAAAACCGTTAAACGCGATGCGAAACCAACCACAAG
CACCGATACAATCAACCACAATGCCTTTAAAGAATGTGTTGATAATGATC
TAGCCGATATATTAAACGATATCAGCGCCTGTACAATAATCCAGAAGAAA
TTATAAAATTATTAAAAAAAAAAAACAAATTCTATGGTGTTTTTCTAATG
CATAAAAAAGGAAATCCCCCTCCAATGGATAAACTAACAAATTATGATAT

TCTTTTTTATGATATAAAAAATTTTTTACAACAATTATTATTTTTTCTTG
TATTAAATGGAATACCTCGTTATAGGATACTTTTTGATGTTGGATTAGGA
TTTGGGAAAAAACATGATCAATCTATTAACCTCTTACAAAATATACTTGT
TTATGATGAGTCTCCTCTTTTTATTGGATATTCAAAAAAAAGATTTATTT
CCCATTGCATGAATGATCAAAATGTTGTAATAAATCCACAACAAAAATTA
CATGATGAACAACAAAATGAAAATAAAATTTTTGTGGACAAATCACACAA
TTGGA

**Table 46: Sample Sequences (Reverse) of *Pfdhps (438)* from Pregnant women Samples**

GGGGGGACCCTACCCCTGTTGTCTATCCTGTAATCCCTGTCGTGTATTTA
TTACAACATCTTGATCTCCTGCAATGGGAAATAAATCTTTTTCTTGAATA
TCCAATAAAAAGCGGATACTCATCATATACACGCACACTTTGTAAGAGCT
TAATAGATTGATCATGCTTCTTCCCAAATCCTAATCCAATATCAAATAGT
ATCCTATAACGAGGTATTCCATTTAATACAAGAAAATTTAATCTTTGTTC
TAAATAATTTTTTATATCATAAACTAGATTATCATAATTTGTTAGTTTAT
CCATTGTATGTGGATTTCCTCTTTTATGCATTAGAACTACACTATAGAAT
TTGTTTTTTTTTTTTTAATAATTTTATAATTTCTGGATTATTTGTCCAACC
CCTAATATCATTTAATATATCAACTAAATCATTATCACCGCATTCATTAA
ATCATTATATTTAATTGTATCAATCCTTATAATTGGTTTCGCATCACATT
TAACAATTTTATTTTTTATTTCATTCCATTCTTTTTGAAATAATTGTAAT
ACAGGTCCTACTAAATCTCTTTCACTAATTTTTGGATTAGGTATAACAAA
AGGACCAGCGGATTCTCCACCTACATCTATAACACTAGCACCTTCATTTA
TTATTTCAAACATTCTTTGAACAGCACGTTTAGGTTCAACAAAAAATACC
AACC

**Table 47: Sample Sequences (Forward) of *Pfcrt* from the Adult Samples**

TGGAAGACATAAAGACATAATCTACAAATAAAGTTCTGTTGAGTTTTCGG
ATGTTACAAAACTATAGTTACCAATCTTTGTTTAAAGTTCTTTTAGCAAA
AATTGTTTCAATTACACATACACTTAAATAAATAATACTTAAAATATAAA
TAAAAATATTATCCTTAATCTCTTTAAAAATAAGTTTAAACACATGAGCA
CATTTACCAAGACAAGAACCTCCACCTAAACGTGAGCCA

**Table 48: Sample Sequences (Reverse) of *Pfcrt* from the Adult Samples**

TGTGCTGTATGTGCTCTGTGTTTAAACTTATTTTTAAAGATATTAAGGAT

AATATTTTTATTTATATTTTAAGCATTATTTATTTAAGCGTATGTGTAAT
TGAAACAATTTTTGCTAAAAGAACTTTAAACAAAATTGGTAACTATAGTT
TTGTAACATCCGAAACTCACAACTTTATTTGTATGATTATGTTCTTTATT
GTTTATTCCTTATTTGGAAATAAAAAGGGAAATTCAA

**Table 49: Sample Sequences of *Pfmdr1* from the Adult Samples**

ACTGCATTTTTTATTAATGACCAATATATAAACCTAAAAAGGAACTGGCA
TATGTAAAAATTGTTATAAATTTCGTACCAATTCCTGAACTCACTTGTTC
TAAATAAAAATCTAAATCAGATCTTAATTTAGATCCAGGATTATTATCAT
GAAATTGTCCATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTAAA
GTTTTTAATATTTTTGATGTAATTACATCCATACAATAACTTGATATCAT
TGATAATATAAATTGTACTAAACCTATAGATACTAATGATAATATTATAG
GATTAATATCATCACCTAAATTCATGTTCTTTAATATTACACCAAACACA
GATATAAAAAAAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGA
TATAAATAATAATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAA
AAAATGATATTTTCTCATTCTTTATTTTTCTAAATAATTCAGCGGTACTT
TTTTTGTTAGTACTC

**Table 50: Sample Sequences of *Pfmdr1* (codon 1034) from the Adult Samples**

CGTTTTTATACAGGAAGCATTTTATAATATGCATACTGTTATTAATTATG
GTTTAGAAGATTATTTCTGTAATTTGATAGAAAAAGCTATTGATTATAAA
AATAAAGGACAAAAAAGAAGAATTATTGTAAATGCAGCTTTATGGGGATT
CAGTCAAAGCGCTCAATTATTTATTAATAGTTTTGCCTATTGGTTTGGAT
CCTTCTTAATTAAAAGAGGTACTATATTAGTTGATGACTTTATGAAATCC
TTATTTACGTTTATATTTACTGGTAGTTATGCTGGAAAATTAATGTCCTT
AAAAGGAGATTCAGAAAATGCAAAATTATCATTTGAGAAATATTATCCAT
TAATGATTAGAAAATCAAATATTGATGTAAGAGATGATGGTGGAATAAGA
ATAAATAAAAATTTAATAAAAGGTAAAGTTGATATTAAAGATGTAAATTT
CCGTTATATTTCAAGACCAAATGTACCTATTTATAAAAATTTATCTTTTA
CATGTGATAGTAAAAAAACTACAGCAATCGTTGGAGAAACAGGTAGTGGA
AAATCAACTTTTATGAATCTCTTATTAAGATTTTATGACTTGAAAAATGA
TCACATTATATTAAAAAATGATATGACAAATTTTCAAGATTATCAAAATA
ATAATAATAATTCATTGGTTTTAAAAAATGTAAATGAATTTTCAAACCAA
TCTGGATCTGCAGAAGATTATACTGTATTTAATAATAATGGAGAAATATT
ATTAGATGATATTAATATATGTGATTATAACTTAAGAGATCTTAGAAACT
TATTTTCAATAGTTAGTCAAGAACCCATTTTATTTTTTATT

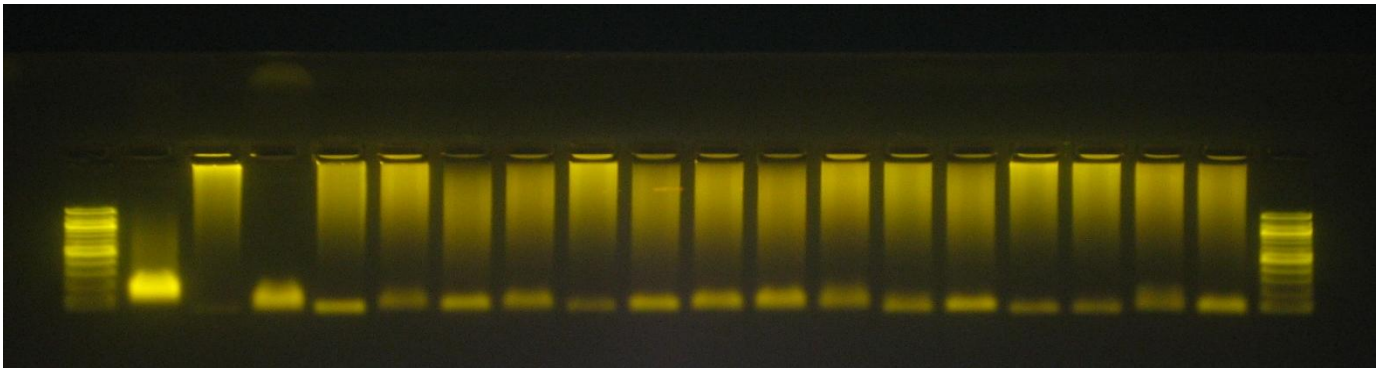
**Table 51: Sample Sequences of *Pfkelch* 13 from the Adult Samples**

CCCGTCCTAGCATTGACAGGTAATTTACACTTGCGCCATTTTTCTCCTCC
TGTAATTATATAAGAATCTGACAATGTGGCAGCTCCAAAATTCATTTTTT
TCTCTGGTACACCATTTAGAAATTGCCATCTTTTATTAAATGGTTGATAT
TGTTCAACGGAATCTAATATGTTATGTTCATTATCAATACCTCCAACAAC
ATATATTTGATTAAGGTAATTAAAAGCTGCTCCTGAACTTCTAGCTTCTA
ATAAGGCATATGGAAATTGTTCCCATTTATTCATTTTTTCTTCATATACT

TCAATAGAATTTAATCTCTCACCATTAGTTCCACCAATGACATAAATTTT
ATTATCAAAAGCAACACACATAGCTGATGATCTAGGGGTATTCAAAGGTG
CCACCTCTACCCATGCTTTCATACGATGATCATATGCTTCTACATTCGGT
ATAATAGAAGAGCCATCATATCCCCCAATACAATAAATTCTACCATTTGA
CGTAACACCACAATTATTTCTTCTAGGTATATTTAAATTACTTGAAACAT
ACCATACATCTCTTAAACGATCATACACCTCAGTTTCAAATAAAGCCTTA
TAATCATAGTTATTACCACCAAAAACGTATAAGAAATTATTCAATACAGC
ACTTCCAAAATAAGCTTTTTTGGTAGACATAGGTGTACACATACGCCAGC
ATTGTTGACTAATATCTAATAATTCCATCGAATTTAAATATTCTACACCA
TCAAATCCACCTATACAAATACTAATGGGAATGGTAAAAAATTTAATACC
ATAAAATTCTGCTTCTTTCACAAAAGGCA

**Table 51: Sample Sequences of *Pfatpase* from the Adult Samples**

ACTCAACTCTCTGGAGCTGCGCGAGTGTCTAGACAGGAGAGGACCCTGGC
GCGGGCTGTAGTGTAAGATGAGTTAAAATTGCTCTACCGCATCTACTGTA
ATGGCTGACTTATCAAAGTCAGTATTATTGTATGTATTAACAATTGTTTG
TTAGACAGAAATTAAGGTATTTGGCCAACGTGATTTTTTTAATATTTATG
TTAAATCTGTGTTGCTTTGGTGGACTTTGTTTGAAAGTAGTGGTGCTTAA
ACTGTTGCTGCTATACCAGGGGGATTGCCAGCAGTCATAACAACTTGTCT
AGCTTTAGGAACAAGAAGAATGGTAAAAAAAAATGCTATAGTAAGAAAAT
TACAAAGTGTTGAGACGTTAGGATGTACAACGGTTATATGTTCTGATAAA
ACAGGTACCCTTACAACAAATCAAATGACAACAACCGTGTTTCATTTGTT
TAGAGAATCTGATTCTTTAACAGAATACCAACTATGTCAAAAAGGGGATA
CCTATTACTTTTATGAAAGTTCAAACTTAACAAATGATATATATGCAGGT
GAATCATCTTTTTTTAATAAATTAAAAGATGAAGGAAATGTTGAAGCTTT
AACGGATGATGGAGAAGAAGGATCAATTGATGAAGCTGATCCATATAGTG
ATTATTTTTCTAGTGATGGTAAGAAAATGAAAAATGATTTAAACAACAAC
AATAATAATAATAATAATAGTAGTAGGAGTGGTGCTAAGGAGGAAGGA



M = Marker, PC = Positive Control, NC = Negative Control

**Fig 1**: Agarose gel picture of Suspected *P.vivax* from Children samples.

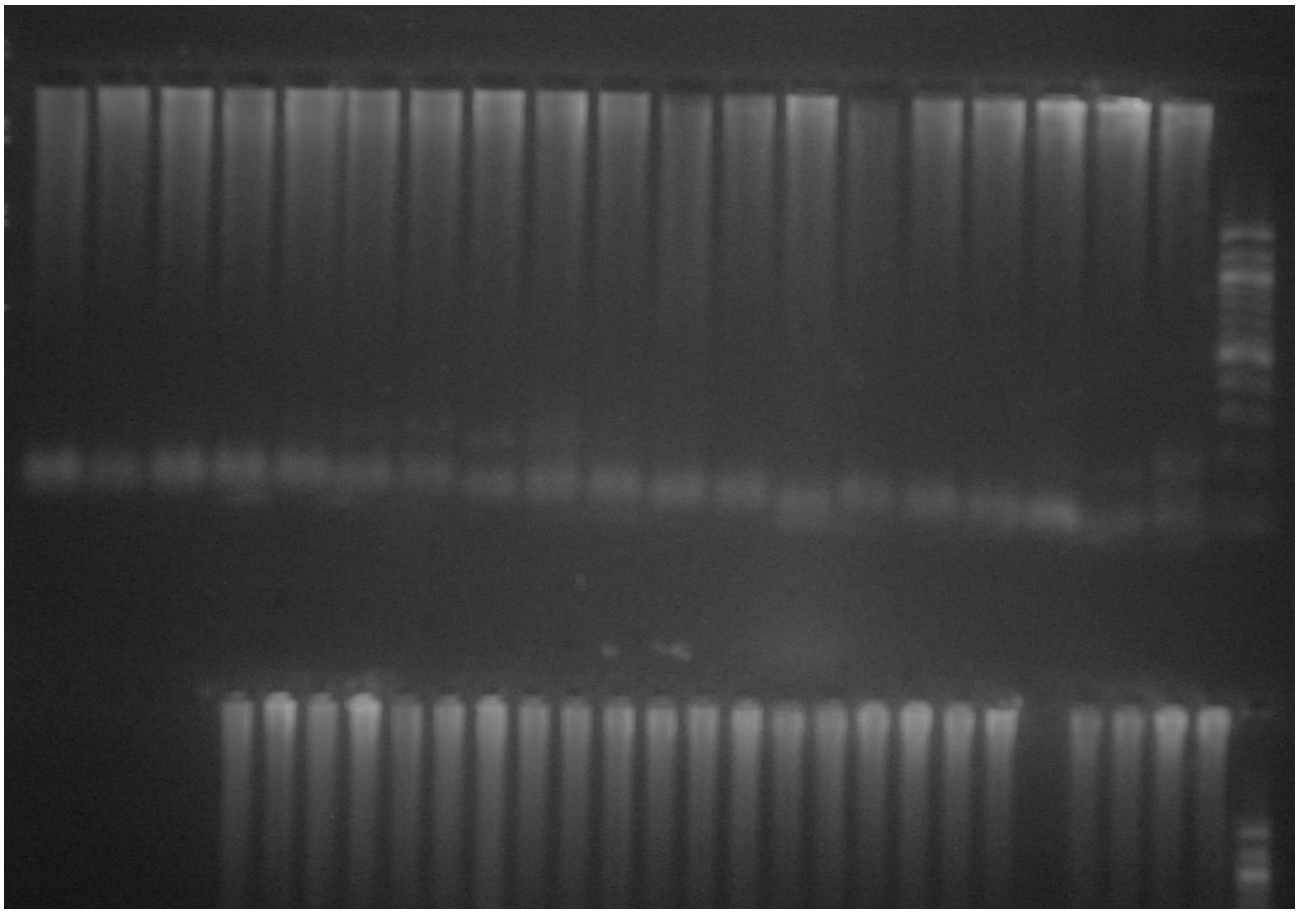


Fig 2: Agarose gel picture of some children samples analysed for *P.vivax*.

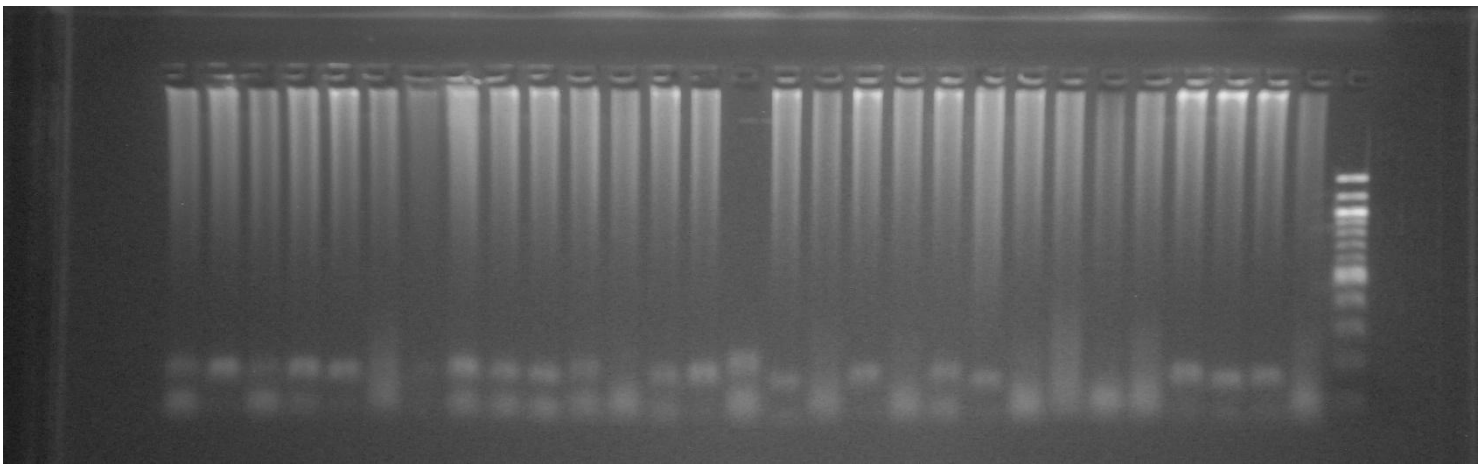


Fig 3: Agarose gel picture of some children samples analysed for *P. malariae*

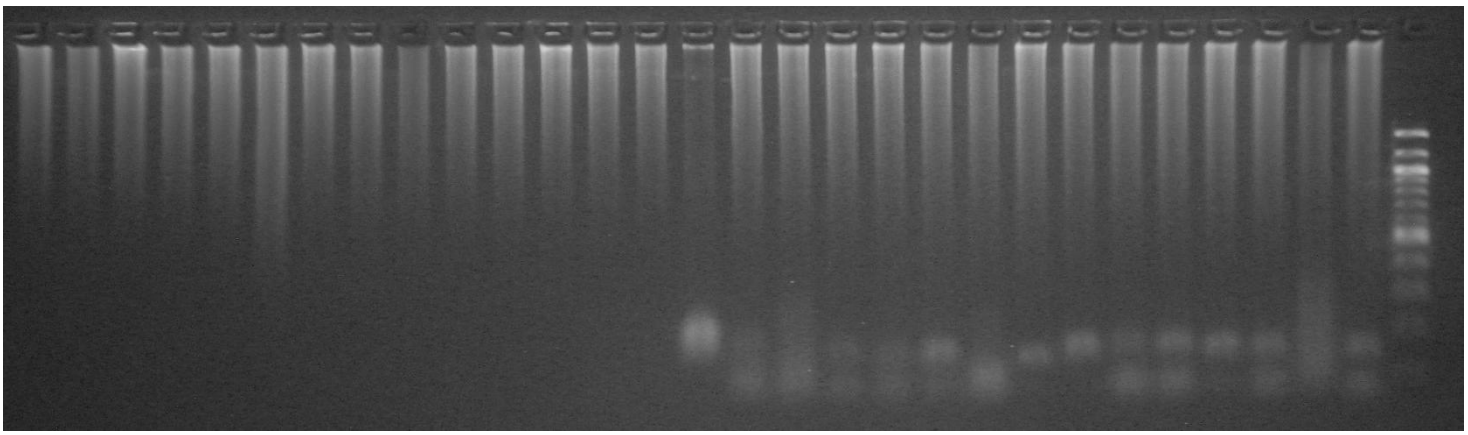


Fig 4: Agarose gel Picture of some children samples analysed for *P.malariae*

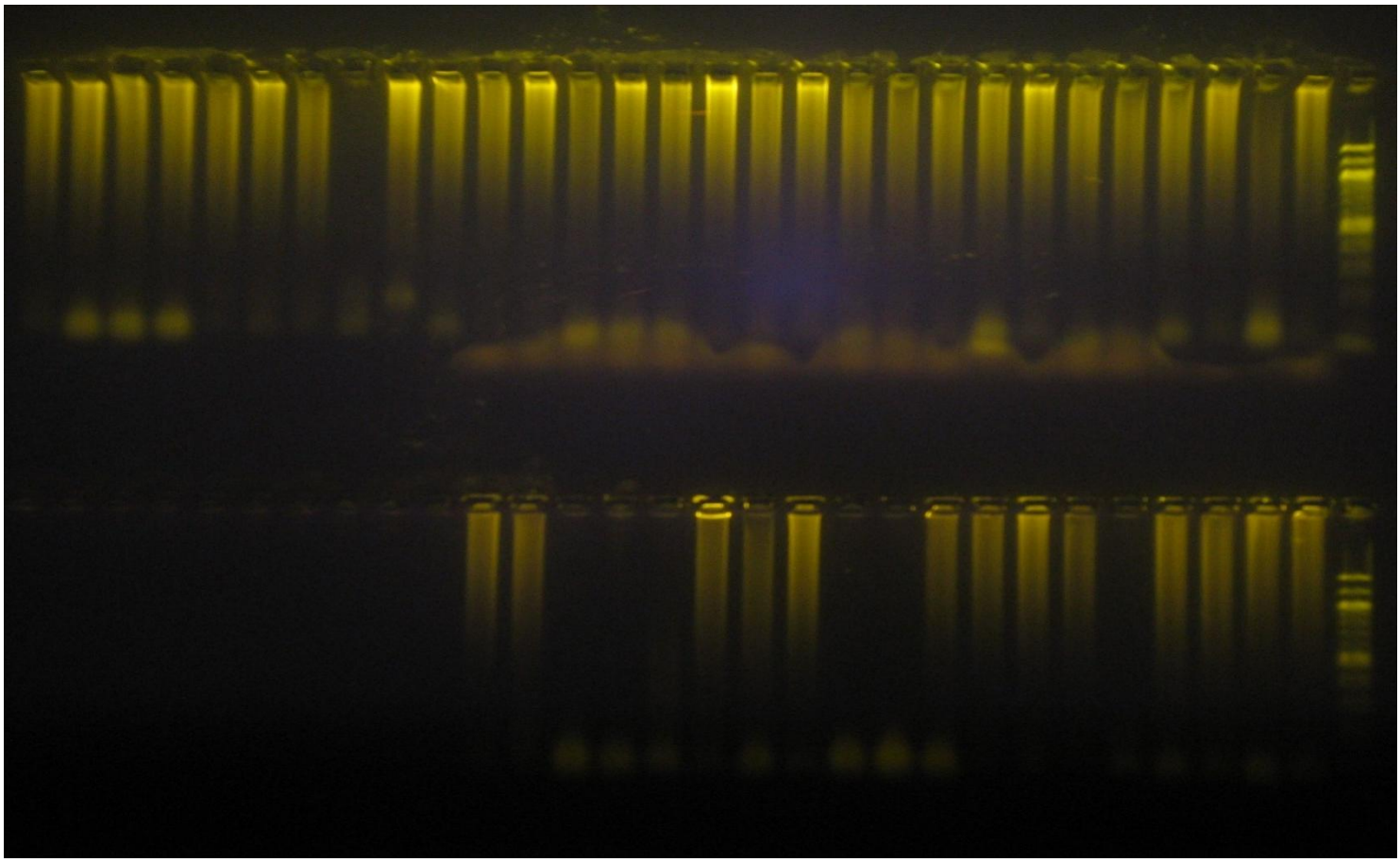


Fig 5: Agarose gel pictures of some children samples analysed for *P.malariae*

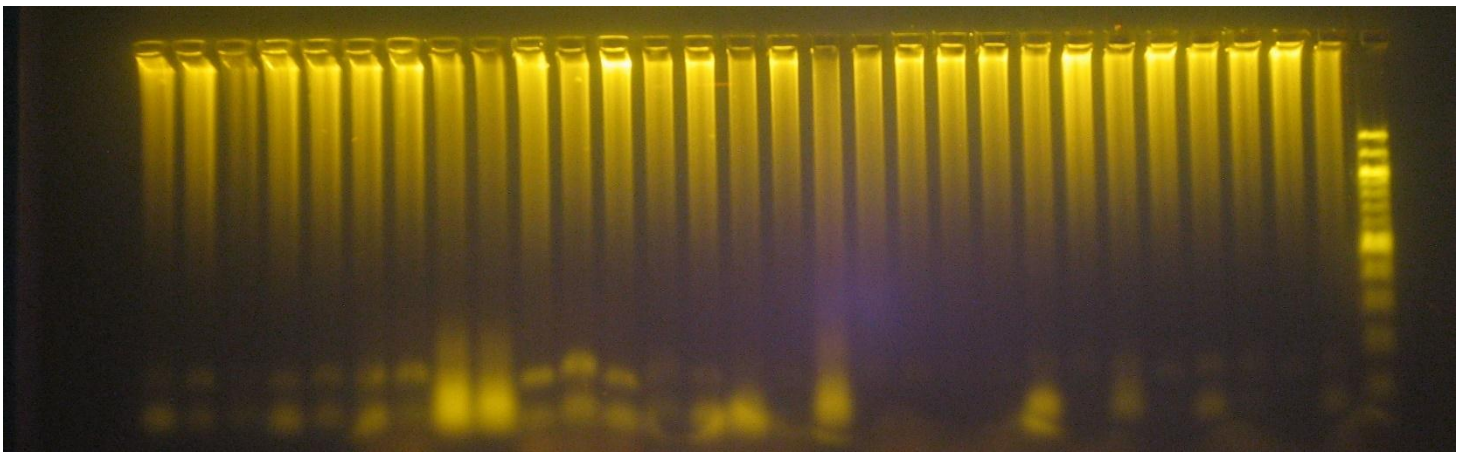


Fig 6: Agarose gel pictures of some children samples analysed for *P.malariae*

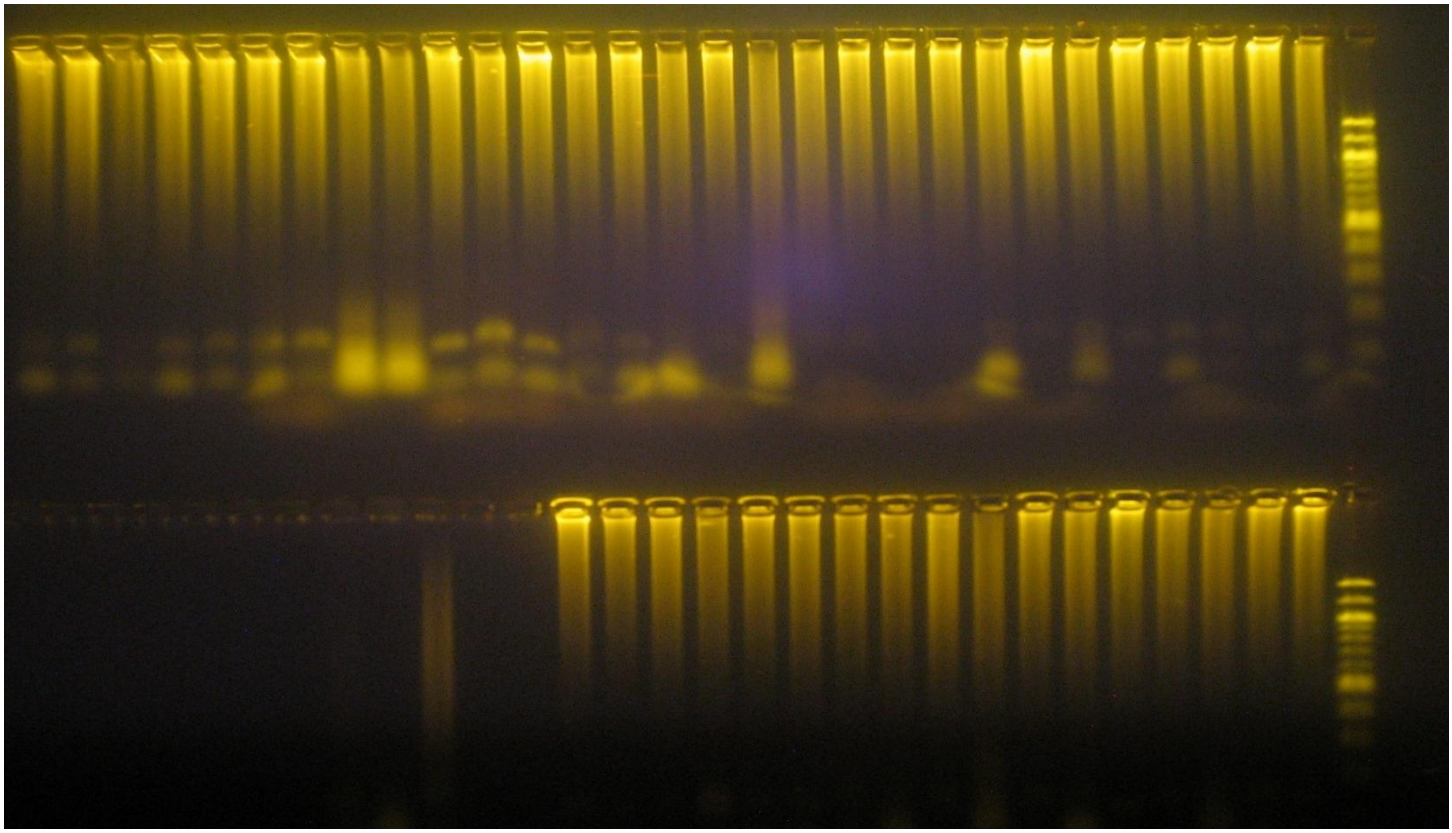


Fig 7: Agarose gel picture of some Children samples of analysed for *P. malariae*

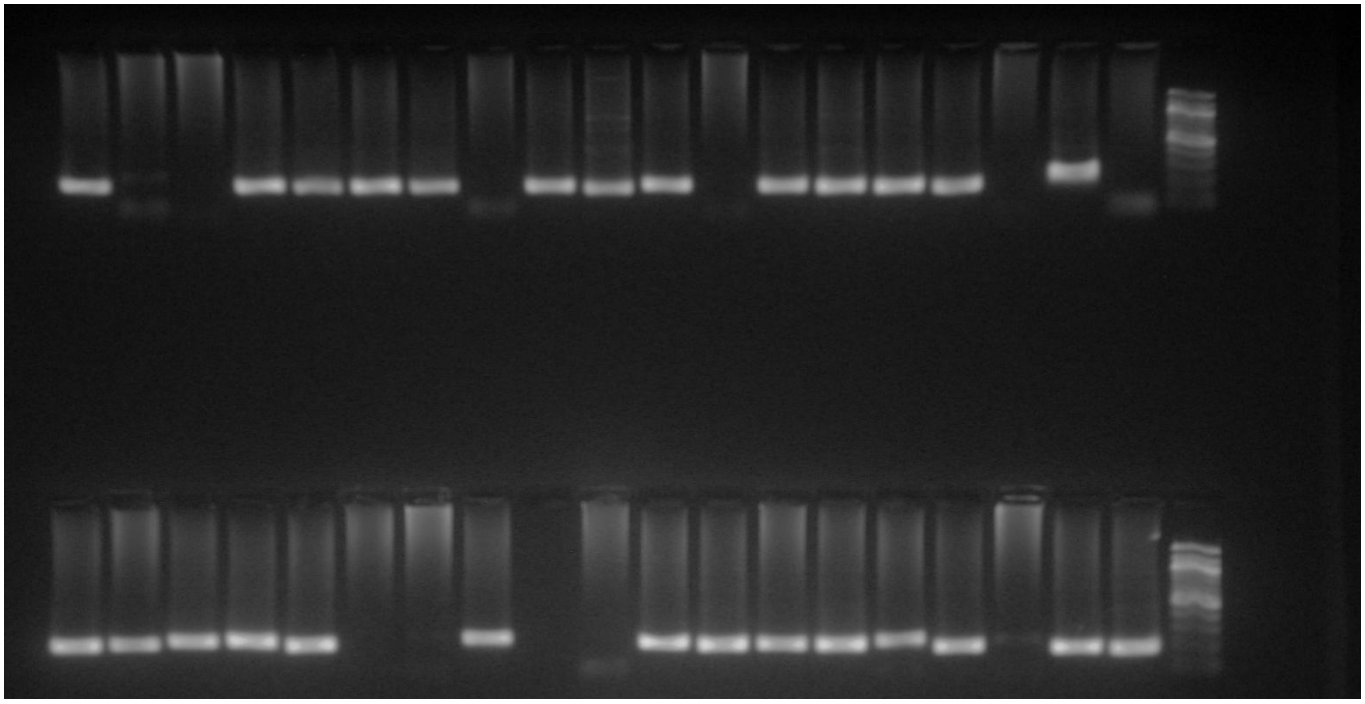


Fig 8: Agarose gel picture of some children samples analysed for *P.falciparum*

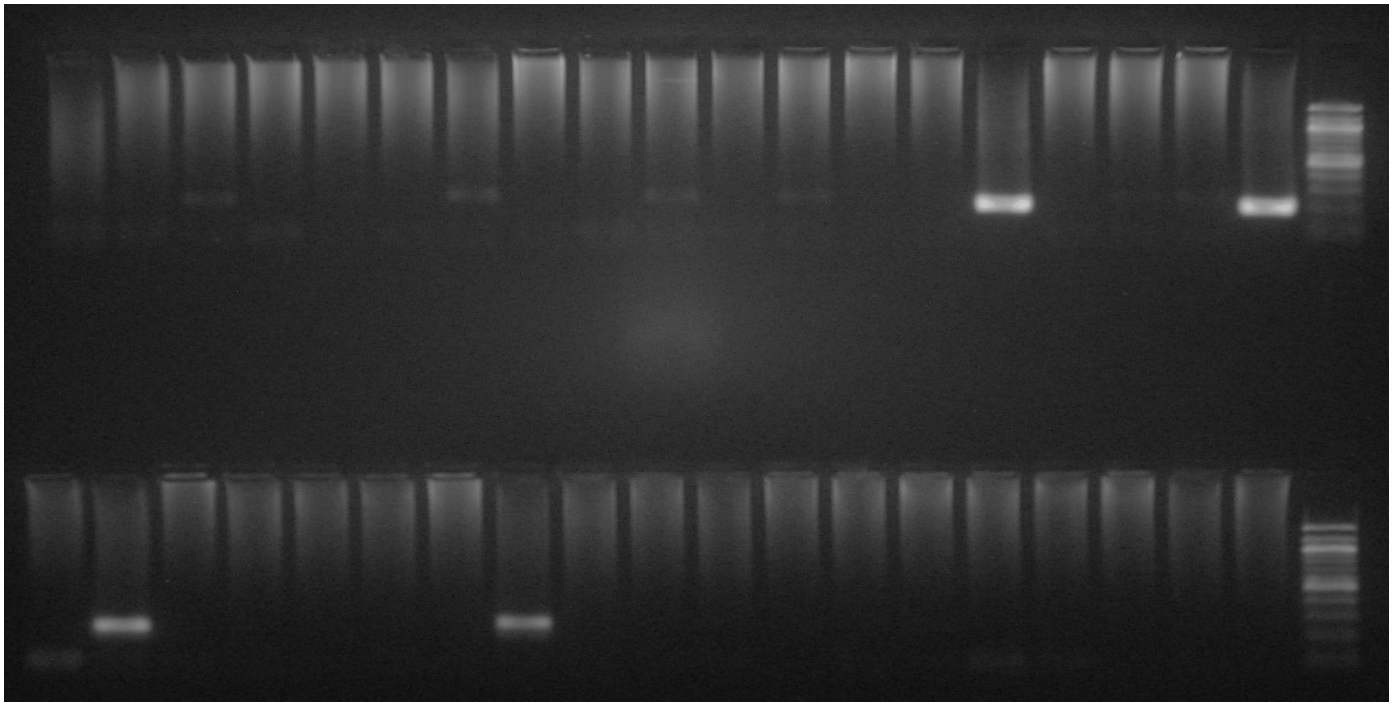


Fig 9: Agarose gel picture of some children samples analysed for *P.falciparum*

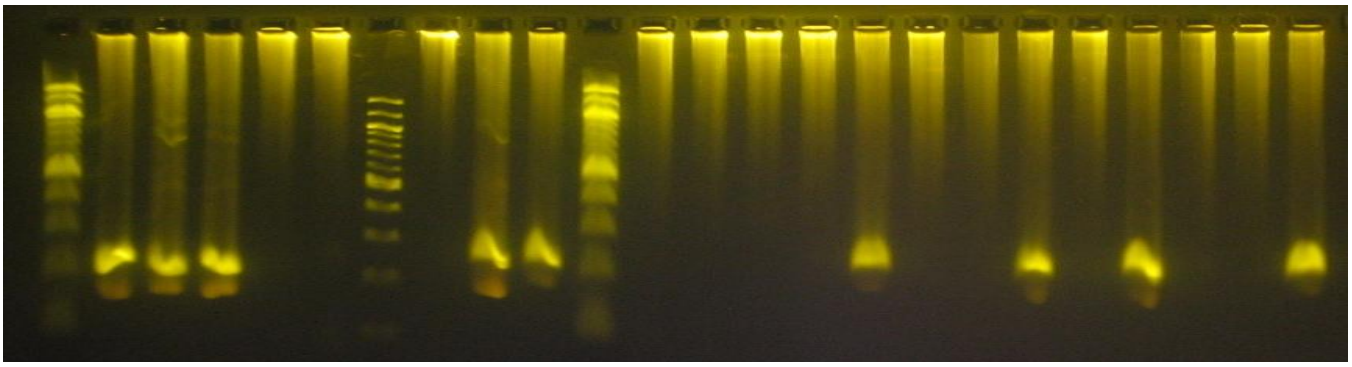


Figure 10: Agarose gel picture of some children samples analysed for *P.falciparum*

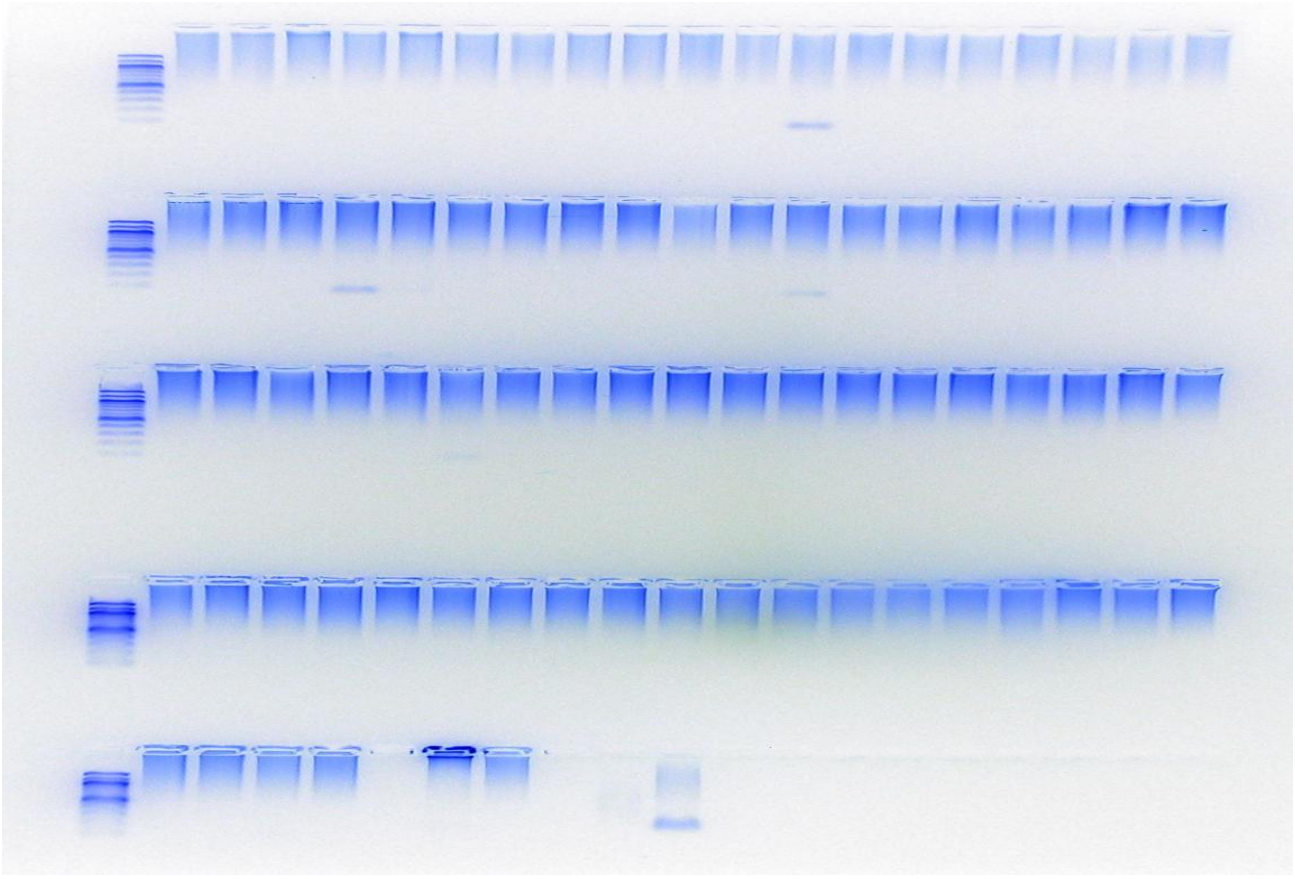


Figure 11: Agarose gel picture of some pregnant women samples analysed for *P.falciparum*

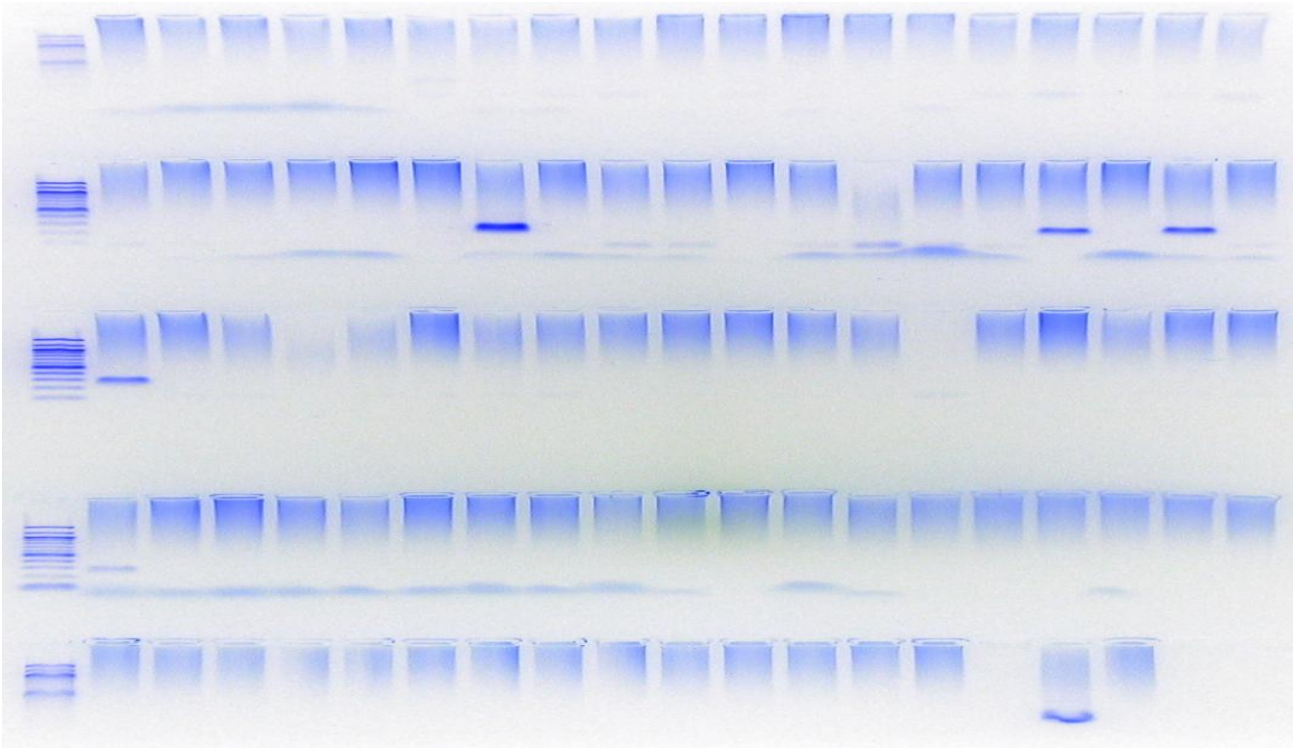


Figure 12: Agarose gel picture of some pregnant women samples analysed for *P. falciparum*

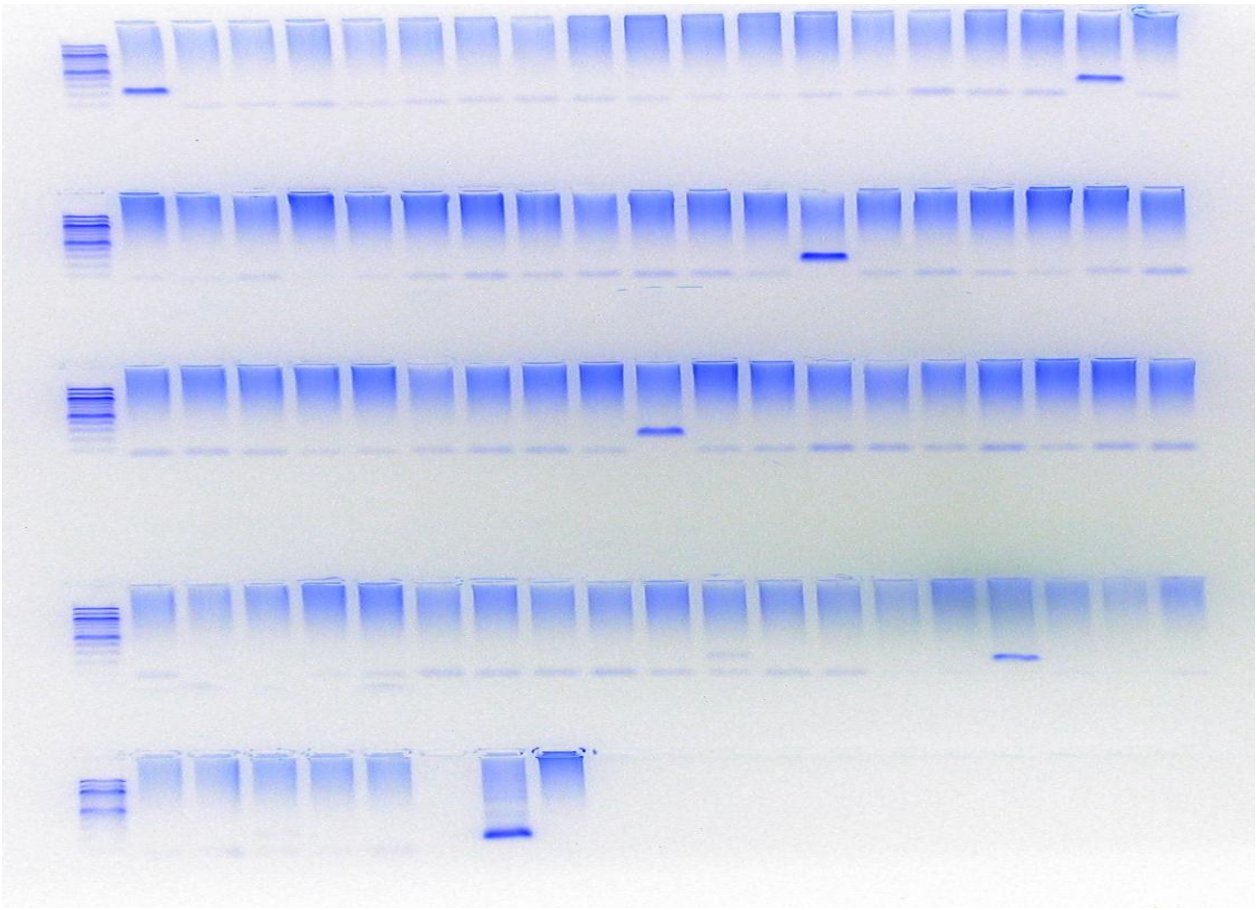


Figure 13: Agarose gel picture of some pregnant women samples analysed for *P. falciparum*

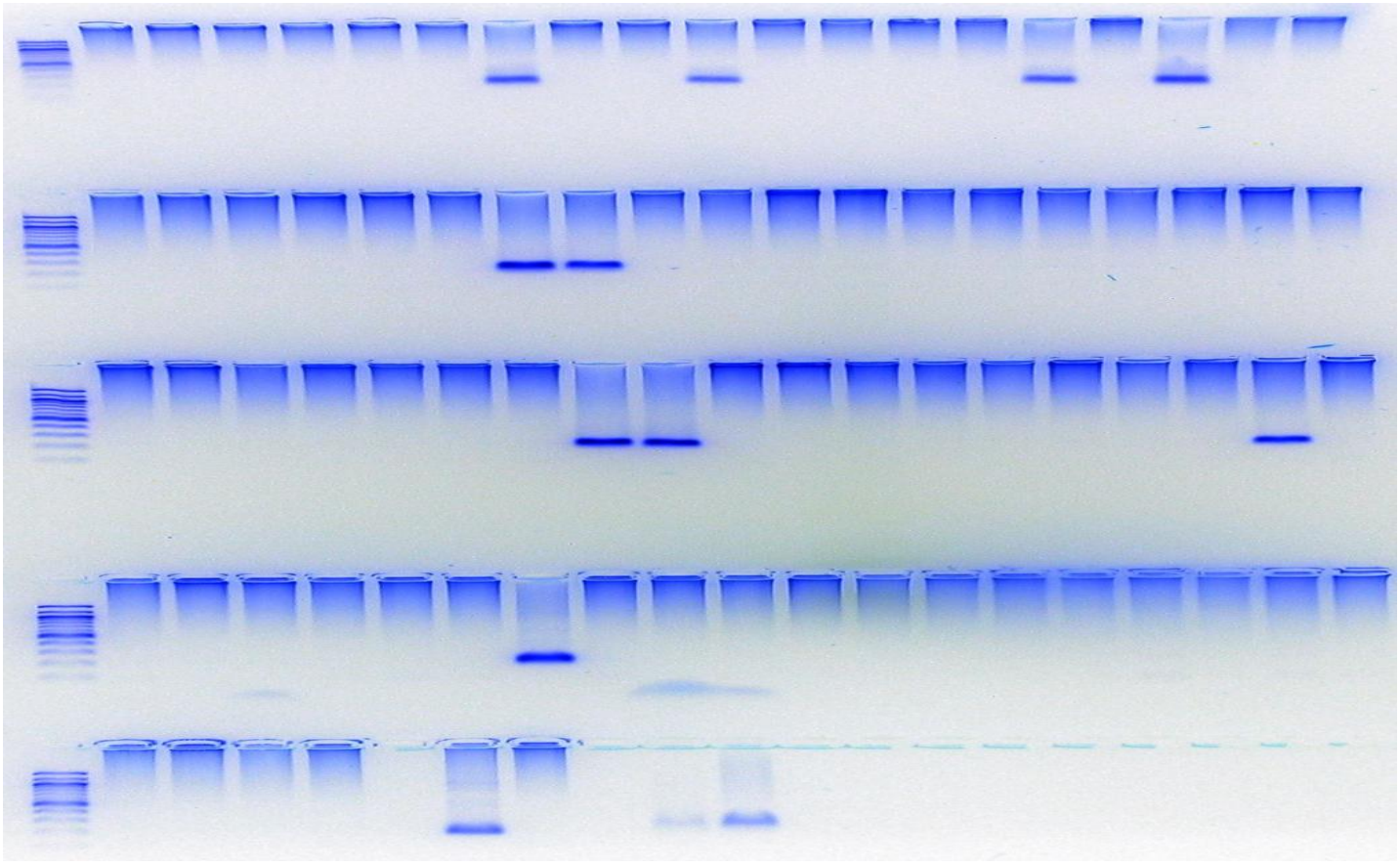


Figure 14: Agarose gel picture of some pregnant women samples analysed for *P. falciparum*

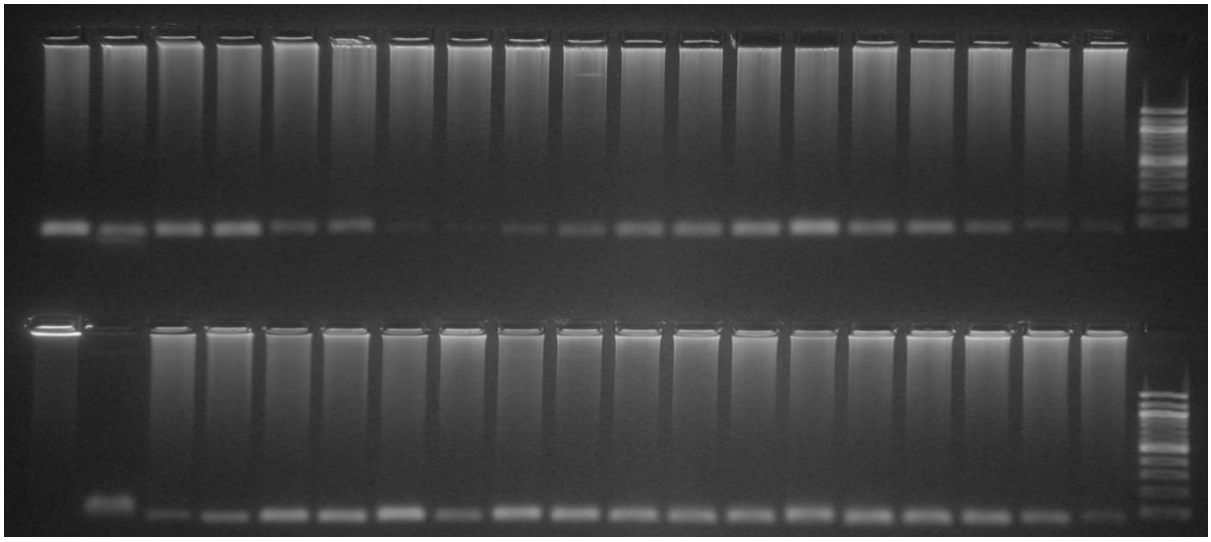


Figure 15: Agarose gel picture of some adult population samples analysed for *P. vivax*

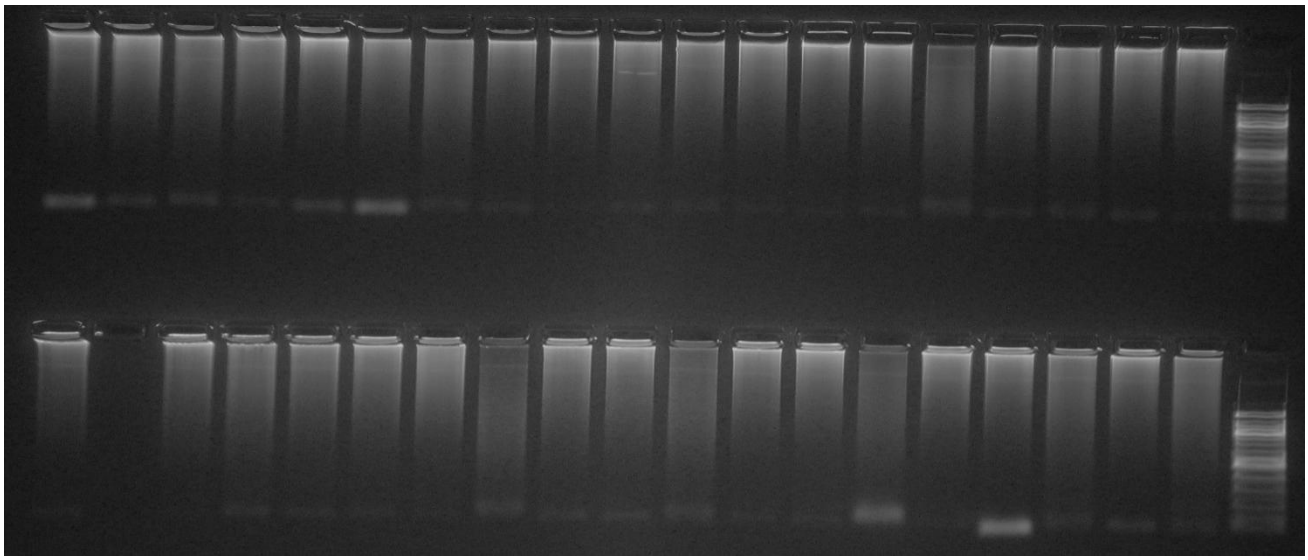


Fig 16: Agarose gel picture of some adult population samples analysed for *P.vivax*

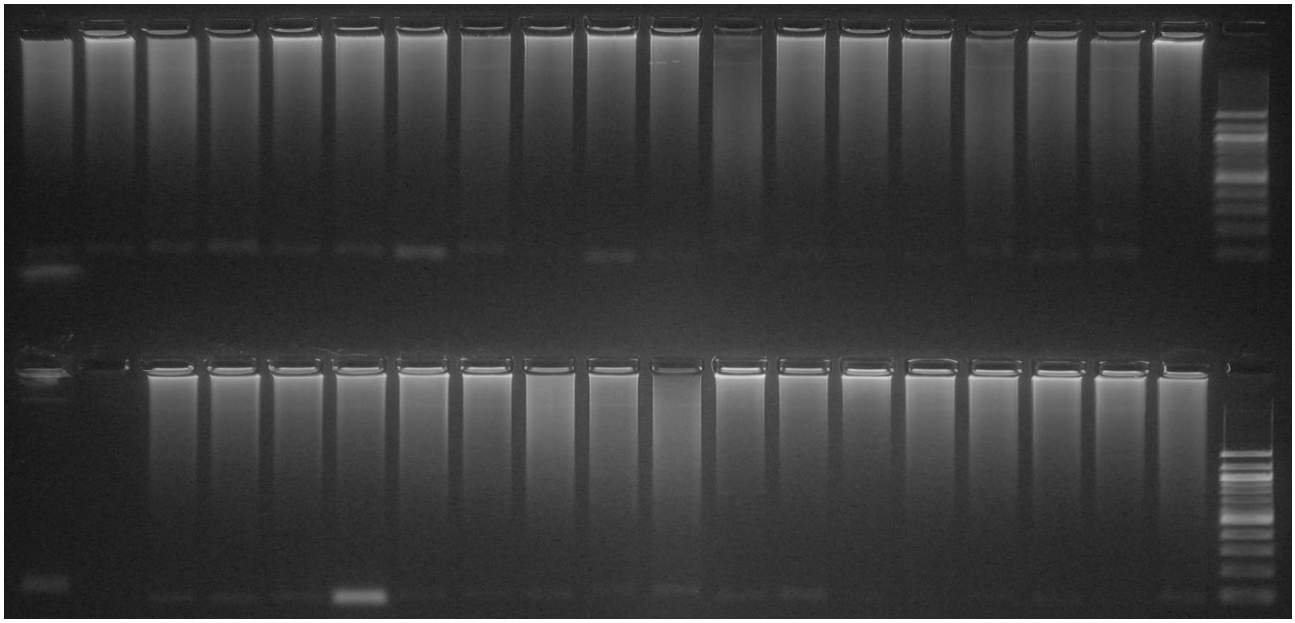


Fig 17: Agarose gel picture of some adult population samples analysed for *P.vivax*

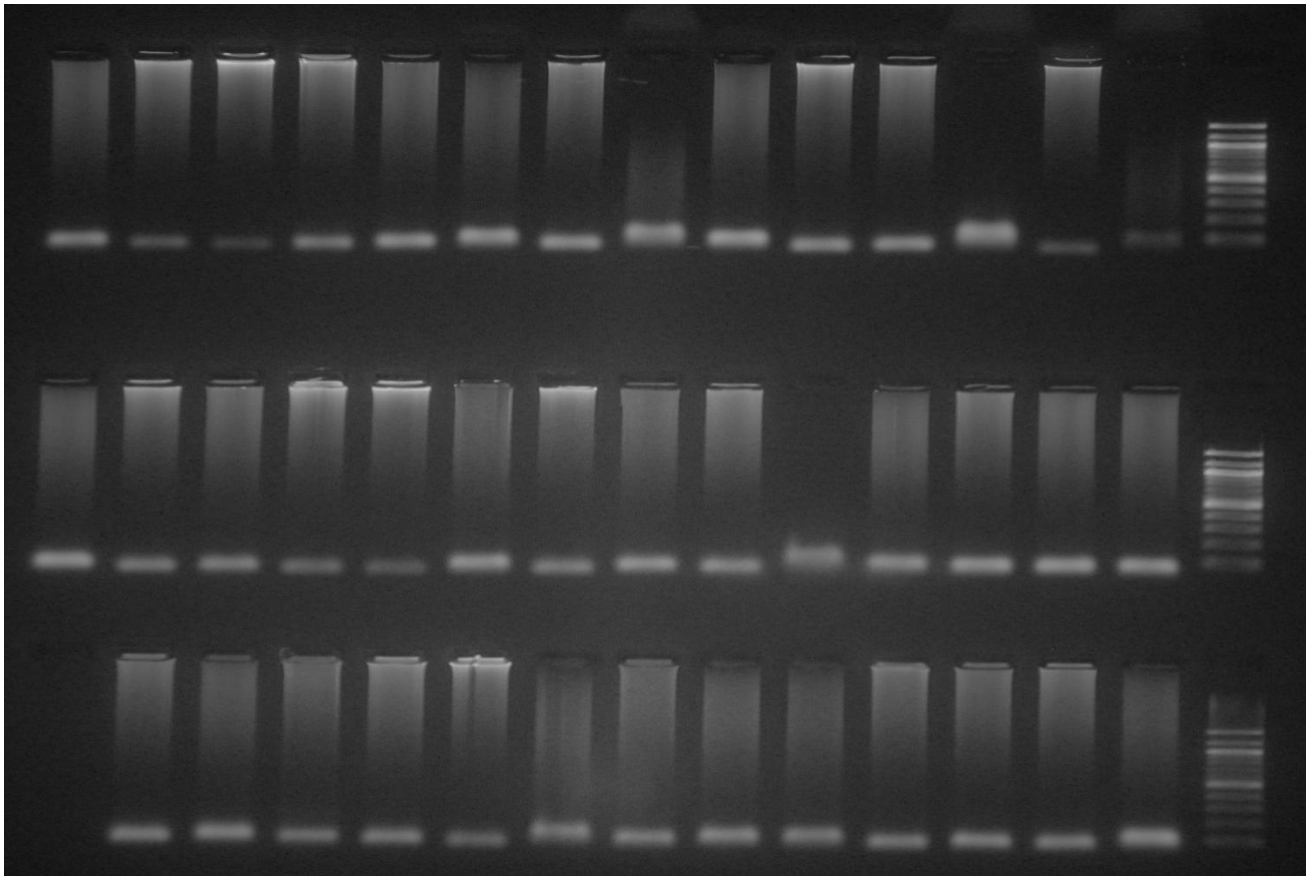


Fig 18: Agarose gel picture of some adult population samples analysed for *P.vivax*

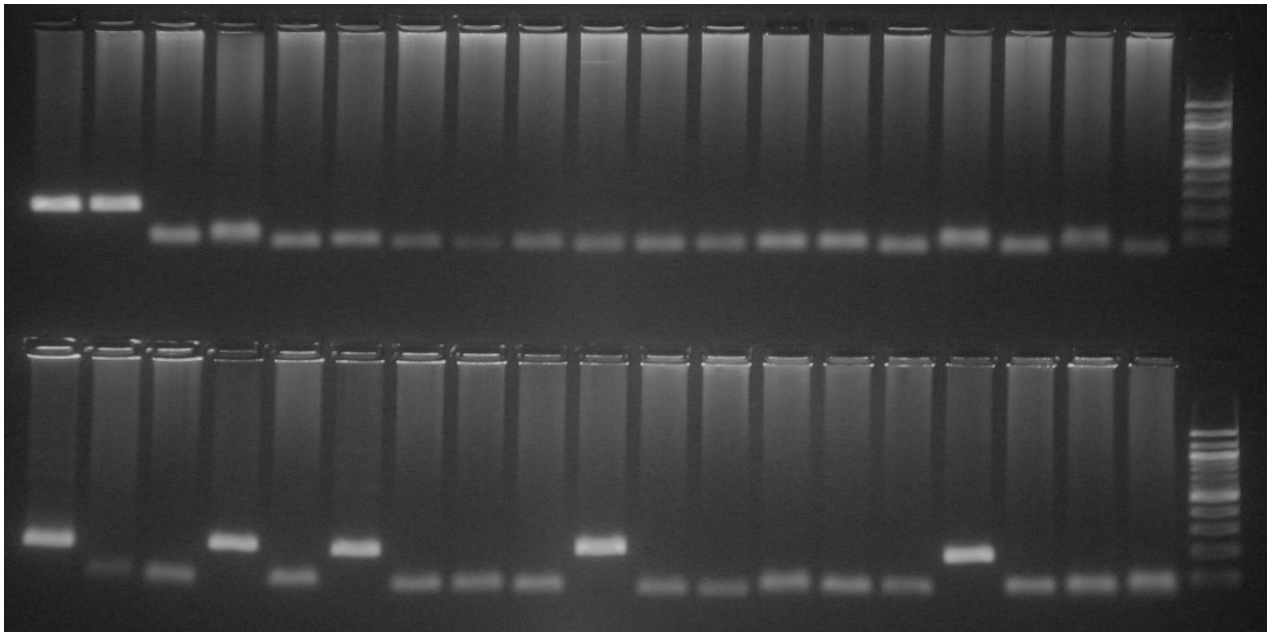


Figure 19: Agarose gel picture of some adult population samples analysed for *P.falciparum*

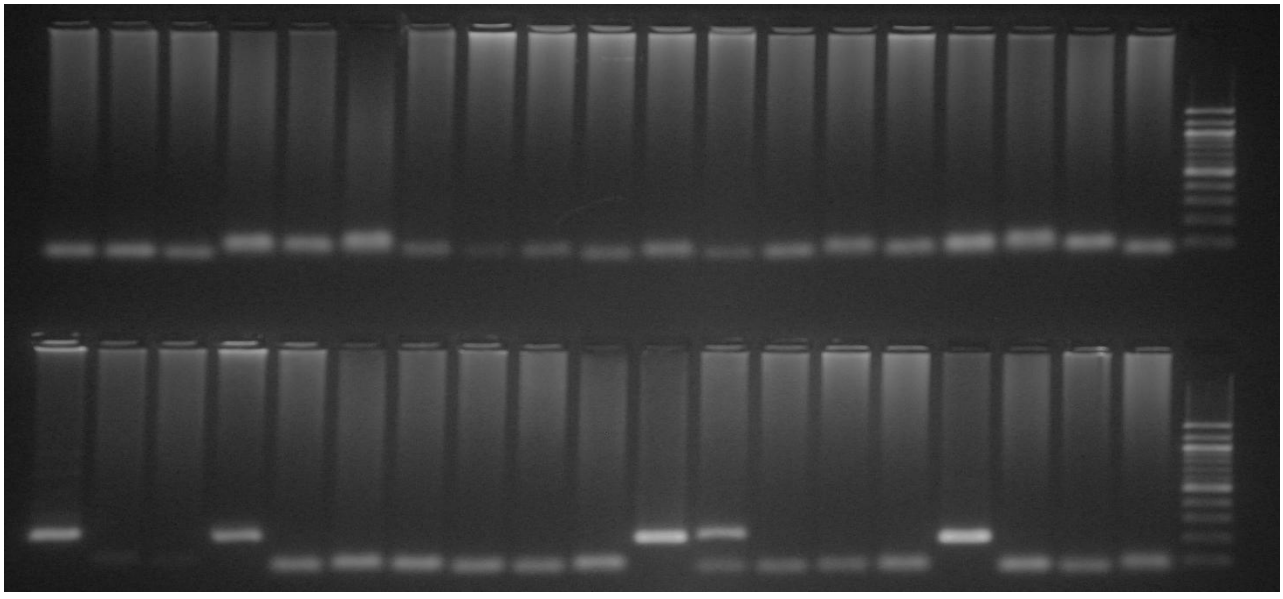


Fig 20: Agarose gel picture of some adult population samples analysed for *P.falciparum*

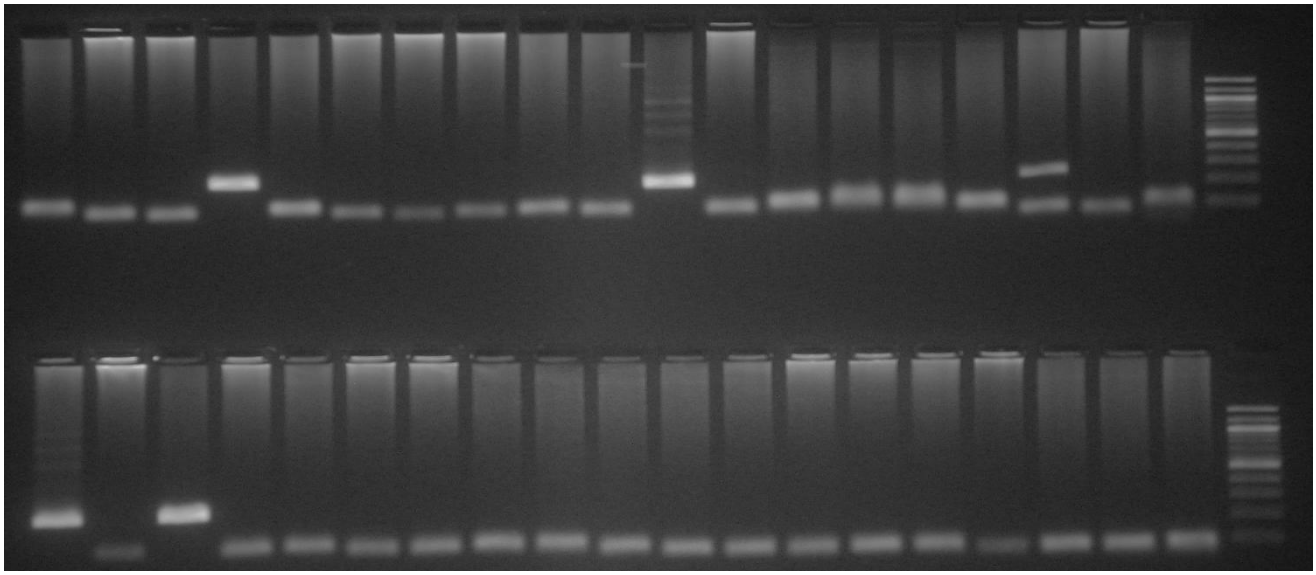


Fig 21: Agarose gel picture of some adult population samples analysed for *P.falciparum*

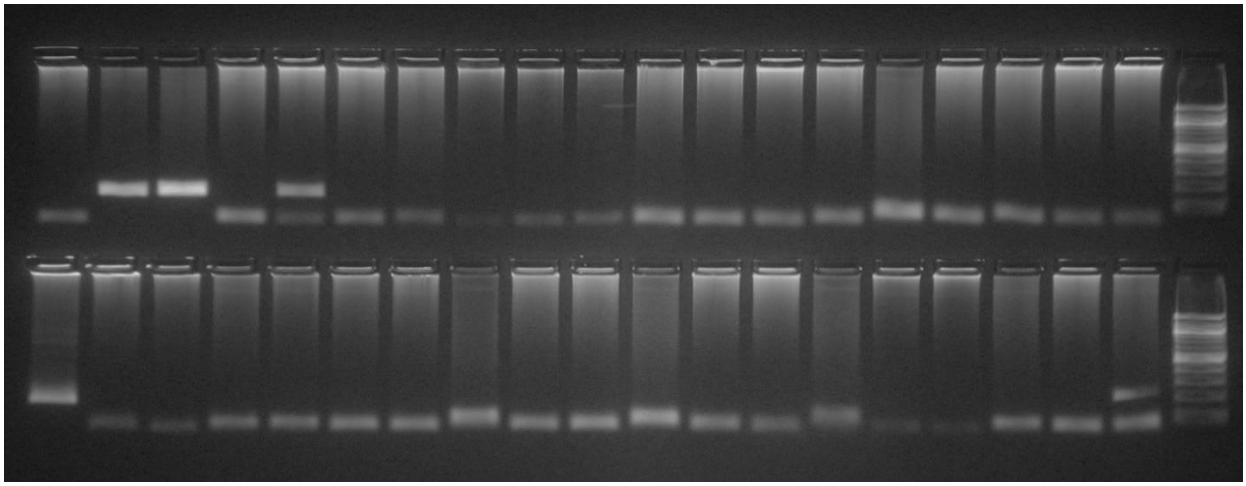


Fig 22: Agarose gel picture of some adult population samples analysed for *P.falciparum*

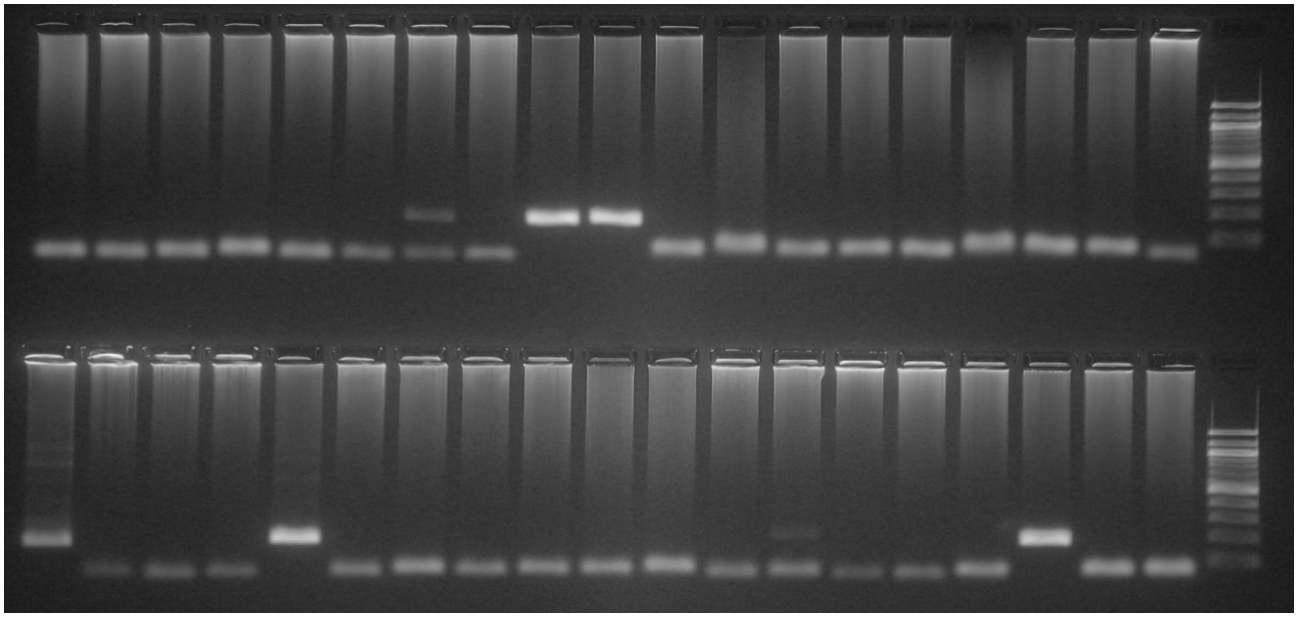


Figure 23: Agarose gel picture of some adult population samples analysed for *P.falciparum*

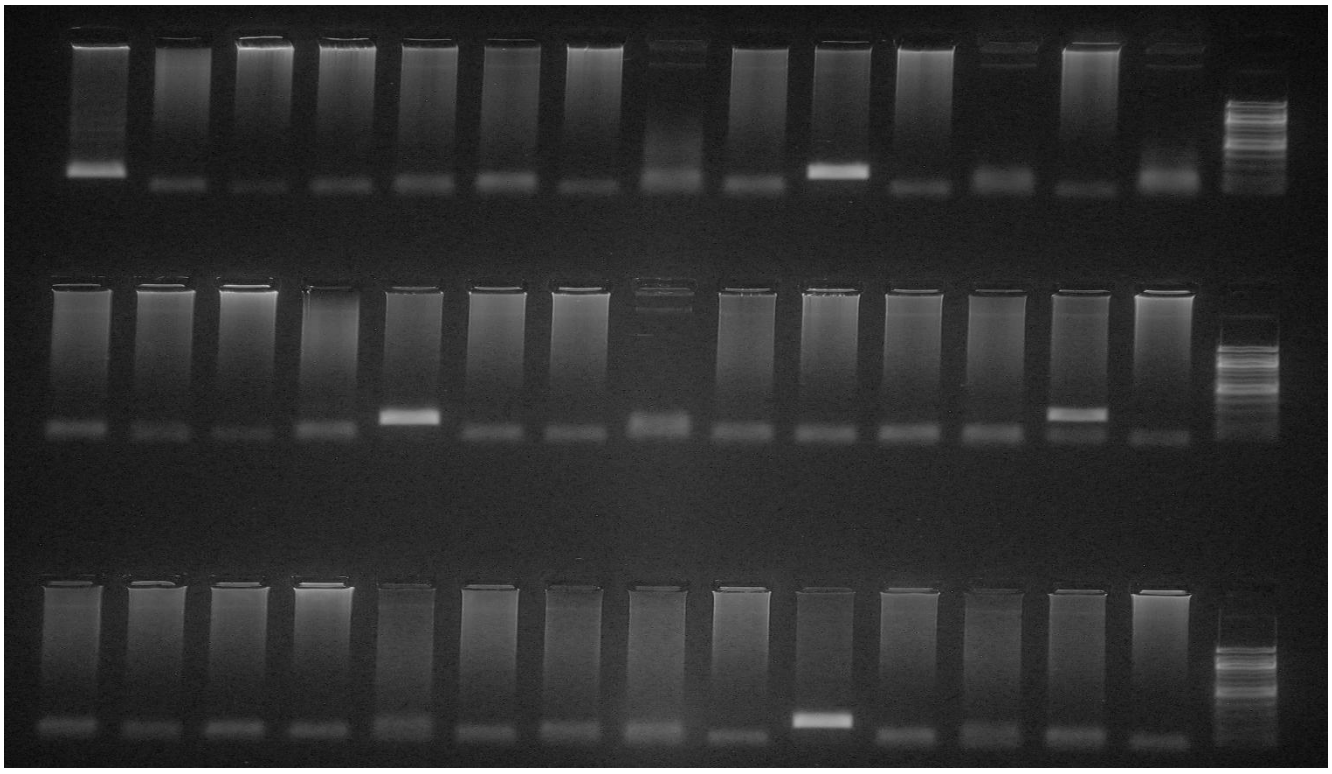


Figure 24: Agarose gel picture of some adult population samples analysed for *P.falciparum*

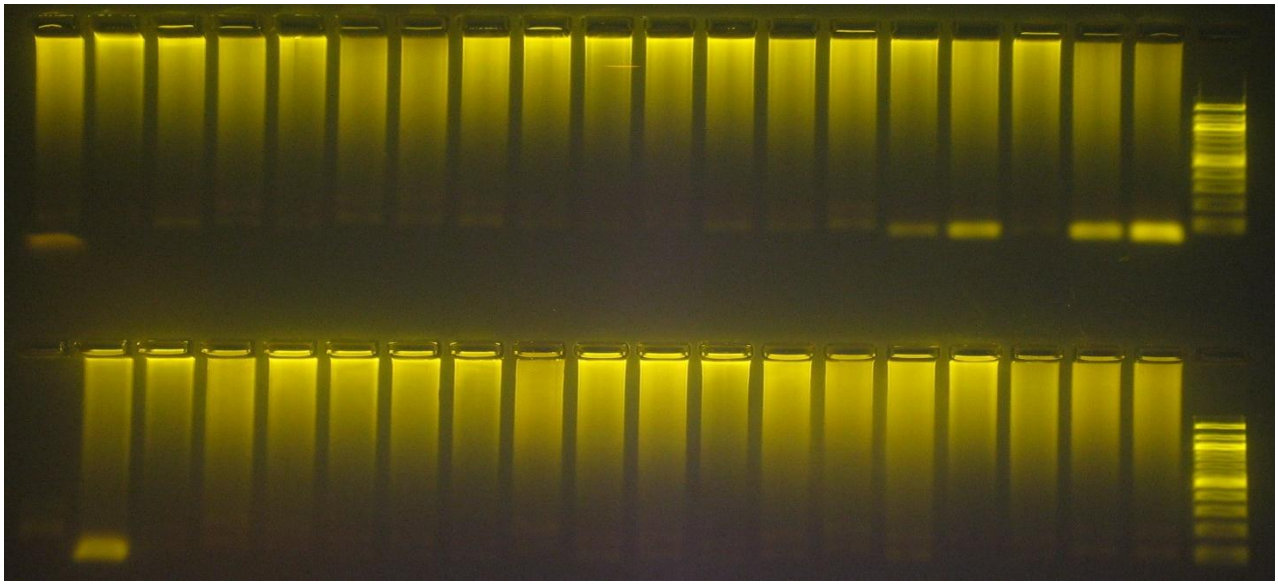


Fig 41: Agarose gel picture of some adult population samples analysed for *P. Malariae*

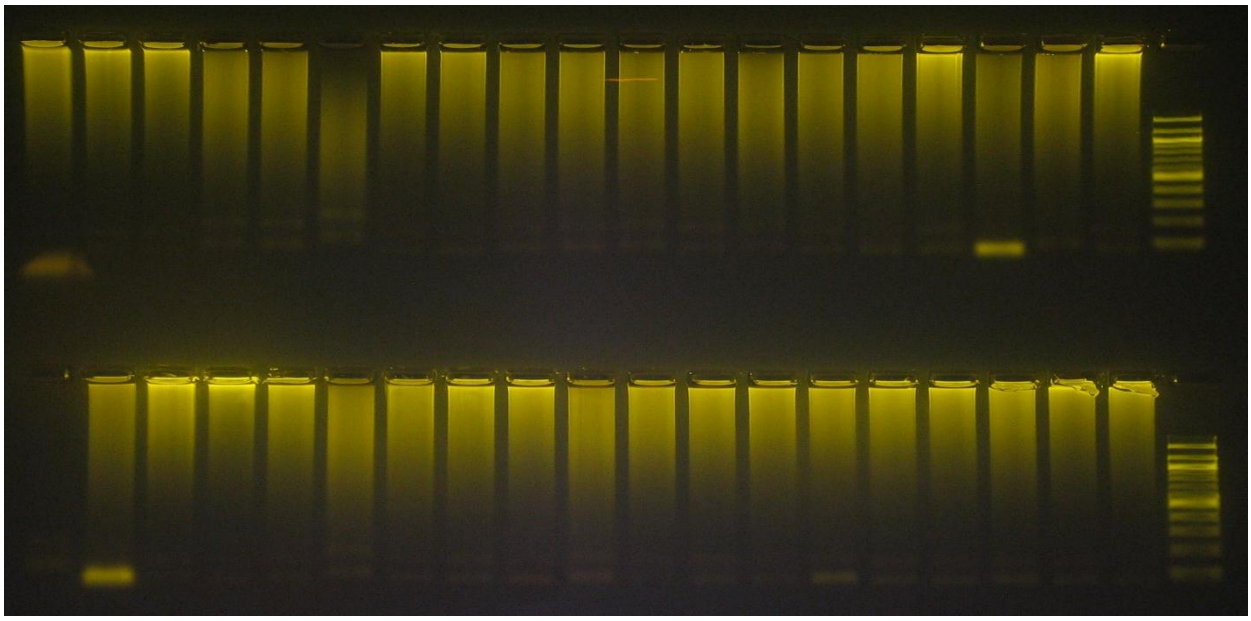


Fig 42: Agarose gel picture of some adult population samples analysed for *P. malariae*

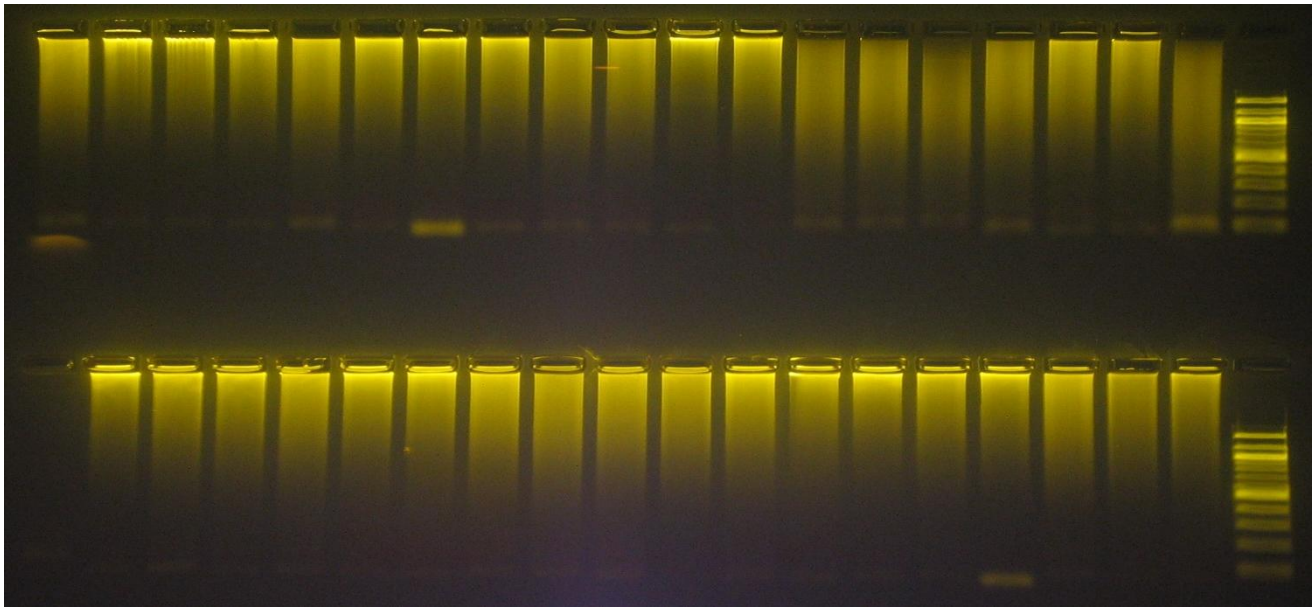


Fig 43: Agarose gel picture of some adult population samples analysed for *P. malariae*



Fig 44: Agarose gel picture of some adult population samples analysed for *P. malariae*